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## CONTENTS



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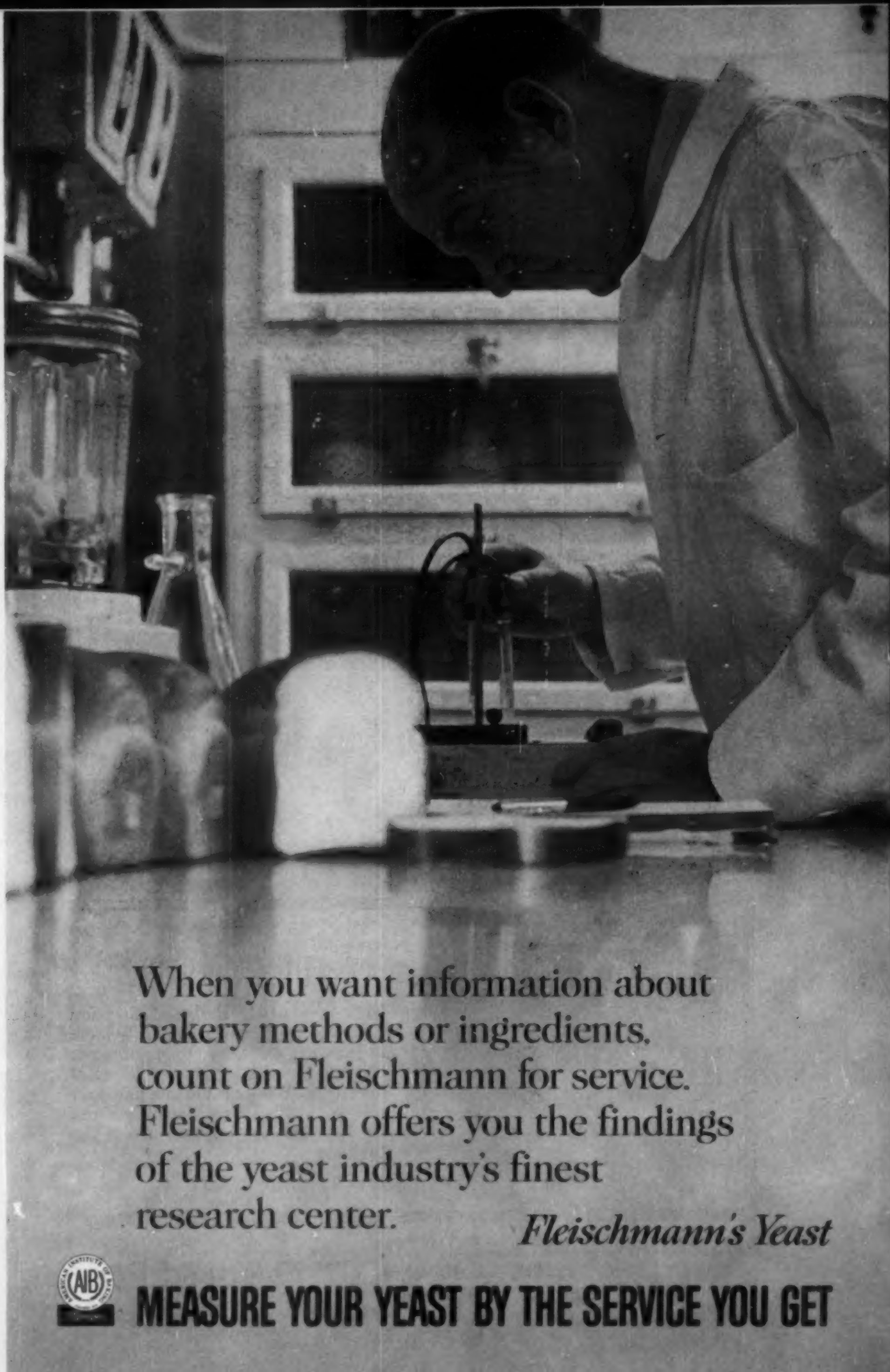
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# CEREAL CHEMISTRY

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## DETERMINATION OF THE NITROGEN CONTENT OF CEREAL GRAIN BY COLORIMETRIC METHODS[1]

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### ABSTRACT

Two modifications of colorimetric methods previously published are presented for the determination of cereal nitrogen content. In method 1, the nitrogen content of white-grained varieties of wheat and barley is determined by a modification of the biuret method (Pinckney). In this method, extraction of the protein and color development occur simultaneously in an alkaline copper tartrate solution. The use of potassium sodium tartrate in place of glycerol in the biuret reagent results in a 13% increase in sensitivity in this method. Those varieties of barley with blue-or black-colored aleurone layers may be conveniently analyzed by method 2 which involves treatment of the extract prepared by the biuret extraction procedure with Folin-Ciocalteu phenol reagent (Lowry *et al.*). The results from both methods are as reproducible as those obtained with the Kjeldahl method of nitrogen determination. (Pinckney: *Cereal Chem.* **26:** 423; 1949. Lowry *et al.*: *J. Biol. Chem.* **193:** 265; 1951.)

The percentage of the total nitrogen content actually extracted into solution in the extraction procedure common to both methods increases as the total nitrogen content increases. Plotting the extracted nitrogen instead of the total nitrogen against the colorimeter reading increases the degree of correlation to only a small extent. The amount of brown-colored material extracted by alkaline solutions from barley and wheat is much greater in the case of barley. The variation in the amount of this extracted brown material is great enough in the case of barley to lessen the degree of correlation between Kjeldahl nitrogen content and the color produced in method 1. The accuracy of method 2 is not affected by this extraneous brown color.

The main factor causing imperfect correlation between Kjeldahl nitrogen content and the color produced in both methods is the variation in the absorbance coefficients of the extracted proteins.

Since, in this laboratory, a large number of determinations of total nitrogen content of both barley and wheat are required each year for plant-breeding purposes, it was decided to adopt colorimetric methods of analysis. Two methods are currently used and both methods are described in this paper.

[1] Manuscript received June 3, 1960. Contribution from the Department of Agricultural Chemistry, Waite Agricultural Research Institute, University of Adelaide, South Australia.

Method 1 is the modification by Pinckney (as reported by Halloran and Moss, 3) of his original method (7), but using a modified extracting solution developed in this laboratory. This method is used for the analysis of red- and white-grained varieties of wheat and white-grained varieties of barley.

Method 2 is an adaptation of the method of Lowry *et al.* (4) and is used for analysis of those varieties of barley which have black or blue hulls, pericarps, aleurone layers, or endosperm outer layers. This method may also be suitable for the analysis of dark-grained varieties of wheats.

A study was made of some factors which could affect the reliability and accuracy of both methods.

## Suggested Methods, and Materials

### Extraction Procedure Common to Both Methods

It has been found convenient to use the same reagents, apparatus, and procedures in both methods until the clarified extract has been obtained.

*Reagent.* The modified biuret reagent used in the common extraction procedure is prepared as follows: 15 ml. of a 10$N$ potassium hydroxide solution and 2.5 g. of potassium sodium tartrate (A.R.) are dissolved in approximately 900 ml. of distilled water; 30 ml. of a 4% copper sulfate pentahydrate solution are then added slowly with continual mixing. Finally, the volume is made to 1 liter.

*Procedure.* Portions of the samples, 500 ± 5 mg., are weighed in a suitably shaped aluminum or plastic scoop on a rapid-weighing balance and then transferred to dry, wide-necked extraction bottles fitted with push-in plastic stoppers. The weighed portions are completely wetted with 2 ml. of carbon tetrachloride. Then 50 ml. of the modified biuret reagent are added and the stoppered bottle shaken on a mechanical shaker for 60 minutes. Portions of the extracts are centrifuged for 10 to 15 minutes at 3,100 R.C.F. (4,500 r.p.m. in an M.S.E. Super Minor centrifuge[2]; Measuring and Scientific Equipment Ltd., 14/28 Spenser St., London S.W.1).

### Method 1[3]

*Procedure.* The clarified extract obtained in the common extraction procedure is read in a suitable colorimeter at 550 m$\mu$ (EEL colorimeter, filter O.G.R.I. or 625; Evans Electroselenium Ltd.,

[2] Mention of proprietary or trade names is not to be construed as an endorsement over other products of a similar nature, but is made for information purposes only.
[3] Refer to Pinckney (3,7).

Harlow, Essex, England) between 105 and 150 minutes after the commencement of shaking.

### Method 2[4]

*Reagents.* Reagent A, 2% sodium carbonate in 0.1*N* sodium hydroxide solution.

Reagent B, 0.5% copper sulfate pentahydrate in 1% potassium sodium tartrate solution. The addition of a few drops of dilute sodium hydroxide solution is necessary to obtain a clear solution.

Reagent C (alkaline copper solution). Mix 50 ml. of reagent A with 1 ml. of reagent B. Discard after 1 day.

Reagent D (diluted Folin-Ciocalteu reagent). Dilute the Folin-Ciocalteu reagent (1) to make it 1*N* in acid. Folin-Ciocalteu reagent (1) can be obtained commercially, or it may be prepared as follows: reflux gently for 10 hours in a 1.5-liter flask a mixture consisting of 100 g. sodium tungstate dihydrate, 25 g. sodium molybdate dihydrate, 700 ml. water, 50 ml. 85% phosphoric acid, and 100 ml. concentrated hydrochloric acid. Add 150 g. lithium sulfate, 50 ml. water, and a few drops of bromine water. Boil the mixture for 15 minutes without the condenser to remove excess bromine. Cool, dilute to 1 liter, and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1*N* sodium hydroxide to a phenolphthalein end point.)

*Procedure.* Transfer, using a Lang-Levy automatic zero micropipet, 0.100 ml. of the centrifuged extracts obtained by the extraction procedure to test tubes, 6 in. by 5/8 in. Add 10 ml. of reagent C and mix. After at least 15 minutes, add 1 ml. of reagent D with immediate and vigorous mixing. Allow at least 30 minutes for full color development before reading in a suitable colorimeter at 760 m$\mu$ (EEL colorimeter, filter O.R.I. or 608).

*Calculation of Results.* A suitable number and range of samples, the nitrogen content of which has previously been determined by a Kjeldahl method on a moisture-free basis, are treated by the appropriate method above. The colorimeter readings are plotted against the corresponding percentage total nitrogen contents on a moisture-free basis and a line of best fit drawn by inspection or alternatively from a numerical analysis of the data. The relation between nitrogen content and absorbance is linear in both methods. The unknown samples are then read off the line, the results being reported as percentage total nitrogen on moisture-free basis. Also, as pointed out (7), the value for nitrogen actually extracted may be substituted

[4] Refer to Lowry *et al.* (3).

for percentage total nitrogen. The reproducibility of both procedures has been found to compare favorably with that of the Kjeldahl method of nitrogen determination, confirming claims made previously (3,7) for the biuret method.

Table I shows the number of samples considered, the range of percentage nitrogen contents (on moisture-free basis) involved, the correlation coefficient, the error[5] of the determination of the nitrogen content by the appropriate colorimetric method assuming that the nitrogen content as determined by the Kjeldahl method is correct, and the equation for the relation between % nitrogen content (on a

TABLE I

ACCURACY OF ESTIMATION OF NITROGEN IN WHEAT AND BARLEY SAMPLES BY METHODS 1 AND 2

| CEREAL AND METHOD[a] | RANGE OF % N (mfb)[b] | CORRELATION COEFF. | ERROR OF COLORIMETRIC DETERMINATION OF NITROGEN[c] (± % N(mfb)) | REGRESSION EQUATION[d] |
|---|---|---|---|---|
| | % | | % | |
| Wheat – 1 | 1.88–2.96 | r = 0.987** | 0.045 | % N(mfb) = 0.883 C− 0.723 |
| Barley – 1 | 1.82–3.22 | r = 0.984** | 0.062 | % N(mfb) = 0.884 C− 1.239 |
| Barley – 1 | 1.16–3.63 | r = 0.997** | 0.062 | % N(mfb) = 5.141 A− 1.042 |
| Barley – 2 | 1.31–3.43 | r = 0.994** | 0.066 | % N(mfb) = 1.087 C− 1.013 |

[a] In each case, 32 samples were treated by the appropriate method.

[b] % N(mfb), percentage nitrogen content on moisture-free basis. The nitrogen content was determined in replicate by a macro-Kjeldahl procedure; the colorimetric values are the result of single determiuations.

[c] The error was determined by the standard linear regression method.

[d] The regression equation showing the relation between %N(mfb) and the colorimeter reading. In these equations, C = EEL colorimeter reading and A = absorbance (Unicam S.P. 600).

moisture-free basis) and absorbance (or colorimeter reading) for some typical standard curves obtained during use of the methods.

## Comments on Procedures

To facilitate the practical use of these methods, the following comments and suggestions are made.

*Grinding.* The samples of whole wheat and barley are ground in a Christie and Norris Junior Laboratory Mill (Thos. Robinson and Son Ltd., Railway Works, Rochdale, Lancashire, England) with a 1-mm. seive and placed in screw-top round sample bottles.

*Mixing.* To allow adequate mixing, the sample should not occupy more than three-quarters of the volume of the bottle. The samples are mixed mechanically for at least 1 hour on a machine which consists of a series of parallel rollers (all driven in the same direction), mounted on a framework which tilts from side to side. Replicate

[5] The error has been determined by the standard linear regression method.

nitrogen determinations on samples taken from the bottle without further mixing, whether by the Kjeldahl method or colorimetric methods, have always shown the usual degree of reproducibility associated with these methods.

*Shaking.* The duration and method of shaking are not critical. The duration may vary from 30 to 120 minutes and the method may vary from gentle mixing to vigorous shaking without affecting the results.

## Discussion of Reagents and Procedures

*Advantages of the Methods.* The considerable economies made in time, capital equipment, and maintenance costs with both methods outweigh, for many purposes, the loss in accuracy when the results obtained are compared with those obtained with the Kjeldahl method. Two operators can carry out over 200 determinations per day when using Pinckney's method (3,7) as set out above in method 1, and about 120 determinations per day when using the procedure of Lowry *et al.* (4) as described in method 2. All the equipment and apparatus used is relatively inexpensive and readily available.

*Choice of Methods.* Varieties of barley grain with blue- or black-pigmented hulls, pericarp, aleurone, or outer endosperm layers must be determined by method 2 since, in the extraction procedure common to both methods, the naturally occurring color is extracted and absorbs strongly at the wave length of maximum absorbance of the copper-protein chelate. This causes large errors in method 1. In method 2 the original extract is diluted about 100 times, and the color developed with the Folin reagent is read at 760 m$\mu$.

Ordinary noncolored barleys may be used to derive the standard curve in method 2 without introducing any error in the determination of the nitrogen content of those varieties of barley with dark-colored grains.

*Moisture Content.* Since the material for analysis for plant-breeding purposes is fully matured in the field before harvest and the moisture content of these samples is not normally required, variations in moisture content have been ignored. The moisture content of the majority of samples will fall very close to the mean of the whole harvest, and the variation of the remainder from the mean does not cause a serious error.

*Lipids and Turbidity.* The extraction bottles must be dry to avoid formation of doughy lumps which are not subsequently wetted with carbon tetrachloride; the samples must be completely wetted with carbon tetrachloride to avoid turbidity from unextracted lipid material.

*The Modified Reagent.* The use of sodium hydroxide instead of potassium hydroxide in the reagent of method 1 is not recommended; the absorption characteristics of the reagents prepared with each alkali are identical, but, when barley or wheat samples are extracted with these reagents, the absorbances of the colors developed with the reagent prepared with sodium hydroxide are only 70% of those of the colors developed with the reagent prepared with potassium hydroxide. Although several experiments were carried out to investigate this reproducible difference, no evidence can be presented to account for this phenomenon.

Pinckney's latest modification of his method (3) has been further modified in this laboratory by the use of potassium sodium tartrate (9,2) instead of glycerol. The use of potassium sodium tartrate has several advantages over glycerol in that it is virtually free of impurities giving rise to precipitation of cuprous oxide, and gives an increase in sensitivity since the copper tartrate chelate has a lower absorbance coefficient than the copper glycerol chelate, and also the copper tartrate chelate solution exhibits maximum absorbance at 675 m$\mu$ compared with 630 m$\mu$ for the copper glycerol chelate solution. This is shown in Fig. 1 where both reagents contained the same concentrations of potassium hydroxide and of copper sulfate. The molar ratio of copper sulfate to sodium potassium tartrate was 1:1.8; that of copper sulfate to glycerol was 1:7.1.

Since, as pointed out by Pinckney (7), copper is removed from the

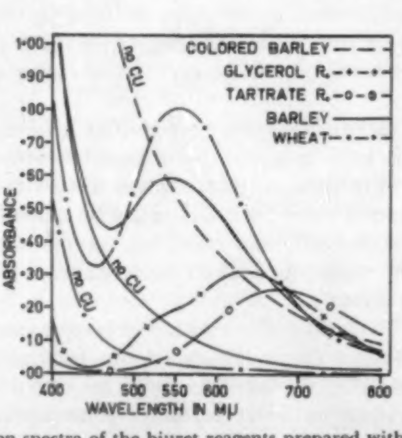


Fig. 1. Absorption spectra of the biuret reagents prepared with potassium sodium tartrate and glycerol, of the biuret colors prepared from a sample of wheat and a sample of barley, and of the extraneous color developed in the absence of copper from these same samples of wheat and barley and also from a colored barley.

copper tartrate complex to react with the protein, the amount of copper tartrate present will decrease proportionally as the protein content of the test solution increases. The absorbance at 550 mμ will be the sum of the absorbances of the copper protein chelate, the remaining copper tartrate chelate, and the extraneous color (see below). The smaller decrease in absorbance of the copper tartrate chelate as compared with the copper glycerol chelate on removal of a given amount of copper by protein results in a 13% increase in sensitivity, or a greater net increase in absorbance at 550 mμ for a given amount of protein. This may be illustrated by an experiment in which 32 samples of wheat were treated with both reagents and the appropriate correlations determined. With the reagent prepared with potassium sodium tartrate, an increase of 1% in nitrogen content (on moisture-free basis) gave an increase in absorbance of 0.222, whereas with the reagent prepared with glycerol the same increase in nitrogen content gave an increase in absorbance of 0.196. The molar ratio of potassium sodium tartrate to copper sulfate (1.8:1) used in this laboratory is adequate to ensure stability of the reagent for several days at least. Since 11 liters of reagent are used per day, greater stability than this is not required. However, higher ratios of tartrate to copper may be used if desired.

*Method 2 – Extraction of Protein*

The method of Lowry *et al.* (4) is about 100 times more sensitive than the biuret method. To avoid undue weighing and sampling errors, it is advisable not to reduce the amount of grain sample taken for analysis below 500 mg. Since the biuret method as employed in this laboratory gives satisfactory extraction of the proteins, the following procedure has been adopted. The biuret color is first developed in the usual manner. After centrifuging, suitable portions (0.100 ml.) are treated by the procedure of Lowry *et al.* (4; see method 2). The biuret reagent is used for the extraction in preference to alkali alone, because a larger proportion of the cereal protein present is soluble in this reagent. Although, theoretically, there is sufficient copper sulfate present in the portion taken to permit full color development in the method of Lowry *et al.* (4; see method 2) the use of reagent C yields more color than reagent A, the increase in absorbance being greater than the increase due to the reagent alone.

*Color Stability.* In method 1, the samples are read between 105 and 150 minutes after the commencement of shaking, as it has been found with both barley and wheat that there is a rapid decrease in absorbance at 550 mμ between 60 and 90 minutes after commence-

ment of shaking; no further change occurs between 105 and 150 minutes, after which a gradual decrease in absorbance takes place. This differs from the findings of Pinckney (7) with wheat, of Robinson and Hogden (8) and Mehl (5) with serum and other proteins, and of Jennings (unpublished) with ovalbumin. In these latter cases, however, the prepared protein solution was subsequently treated with alkaline copper sulfate, whereas in the method described here the extraction of protein and color development occur simultaneously.

It is necessary when using method 2 to adhere fairly closely to a convenient time schedule to avoid batch-to-batch variations in the absorbance of the developed colors.

*Instruments Used.* The results reported in this paper have been obtained using either a Unicam SP600 Spectrophotometer (Unicam Instruments Ltd., Arbury Works, Cambridge, England) or an EEL Colorimeter. All readings in the Unicam SP600 Spectrophotometer have been made using cuvets of 2-cm. light path with distilled water in the reference cuvet, and in the EEL Colorimeter with the standard tubes (3⅛ in. by ⅝ in. diameter) supplied with the instrument, again with distilled water in the reference tube.

## Investigation and Discussion of Factors Affecting Accuracy

*Extraneous Color.* When plant materials are extracted with alkaline solutions a certain amount of extraneous brown color is usually associated with the alkaline extracts. This extraneous brown color is usually due to hemicelluloses, lignin, polyphenolic compounds, and natural pigments in the extract. The amount of this extraneous brown color in alkaline extracts of barley is much greater and more variable than in alkaline extracts of wheat. In neither barley nor wheat is the amount of this color related to total nitrogen or extractable nitrogen content. The extraneous color was developed by extraction of samples with $0.15N$ potassium hydroxide solution instead of the biuret reagent, using the recommended procedure. When read in cuvets of 2-cm. light path at 550 $m\mu$, the range of the absorbances of the extraneous color of wheat was 0.029 to 0.041, with an average value of 0.035.

The variability in this color in barley is large enough to lessen the degree of correlation obtained in method 1. The degree of correlation is improved and the error of the determination of nitrogen content by method 1 is decreased if the absorbance of this extraneous color is subtracted from the absorbance of the biuret color and the difference (i.e., the color due to the copper protein chelate and the unreacted reagent) plotted against total nitrogen content, as shown

in Table II. Table II shows some typical absorbance values for the biuret color, the extraneous color, and the difference between these for white-grained samples of barley, and also values for two barleys with dark-colored grains. The values for the colored barleys are omitted from the calculations for the correlation coefficients, the errors of the determinations, and the equations showing the relationship between nitrogen content and absorbance. The effect of this extraneous

TABLE II
EFFECT OF EXTRANEOUS COLOR ON ACCURACY OF METHOD 1

| | Barleys %N(mfb)[a] | Absorbance: Biuret Color | Absorbance: Extraneous Color | Absorbance: Biuret Minus Extraneous Color |
|---|---|---|---|---|
| | % | | | |
| White-grained | | | | |
| | 1.16 | 0.429 | 0.137 | 0.292 |
| | 1.45 | .476 | .109 | .367 |
| | 1.75 | .550 | .132 | .418 |
| | 1.82 | .526 | .117 | .409 |
| | 2.16 | .604 | .125 | .479 |
| | 2.21 | .617 | .110 | .507 |
| | 2.34 | .673 | .148 | .525 |
| | 2.51 | .658 | .125 | .533 |
| | 2.60 | .687 | .127 | .560 |
| | 2.74 | .721 | .124 | .597 |
| | 3.02 | .777 | .117 | .660 |
| | 3.22 | .798 | .116 | .682 |
| Colored[b] | | | | |
| | 2.43 | .805 | .382 | .423 |
| | 2.97 | 1.240 | 0.531 | 0.709 |

| | Corr. Coeff. | Error of Colorimetric Determination of Nitrogen[c] (± %N(mfb)) | Regression Equation[d] |
|---|---|---|---|
| | | % | |
| Biuret A vs. % N (mfb) | r = 0.993** | 0.079 | % N (mfb) = 5.350 A − 1.103 |
| (Biuret A − extraneous A) vs. % N (mfb) | r = 0.996** | 0.061 | % N (mfb) = 5.300 A − 0.415 |

[a] %N(mfb), percentage nitrogen content on moisture-free basis.
[b] Excluded from correlation coefficient and other calculations.
[c] The error was determined by the standard linear regression method.
[d] The regression equation showing the relation between %N(mfb) and the absorbance (A) values in the calculated line of best fit.

color becomes negligible in the method of Lowry *et al.* (4), as set out in method 2. This is demonstrated by the fact that when 0.15*N* potassium hydroxide extracts of barley and wheat and a preparation of ovalbumin are serially diluted and these dilutions are treated by the method of Lowry *et al.* (4), as set out in method 2, the resultant

plots of absorbance against nitrogen content very nearly coincide.

Typical absorption spectra of the extraneous color (i.e., the color extracted by alkali in the absence of copper) from wheat, barley, and a barley with a black aleurone layer are shown in Fig. 1. Also included in Fig. 1 are the absorption spectra of the developed biuret color (using the modified reagent recommended above) from the same samples of wheat (2.96% nitrogen, moisture-free basis) and barley (1.86% nitrogen, moisture-free basis). The influence of the extraneous color on the shape and point of maximum absorption of the absorption spectra of the biuret colors derived from barley and wheat is shown in Fig. 1.

*Amount of Nitrogen Actually Extracted.* The amount of nitrogen actually extracted (as determined by a micro-Kjeldahl procedure) by the recommended extraction procedure from 500-mg. portions (of approximately 10% moisture content) of barley samples of varied total nitrogen contents was subtracted from that calculated to be present (allowance being made for the moisture content).

The unextracted nitrogen was calculated as percentage of the original sample on a moisture-free basis. This figure was relatively constant and was not related to total nitrogen content. The unextracted nitrogen content covered the range 0.10 to 0.31% with an average value of 0.18%. (Any amide nitrogen liberated and lost from the extracts would be included in the figure for unextracted nitrogen.)

This experiment was repeated, with omission of the copper sulfate and potassium sodium tartrate from the reagent. Similar results were obtained, except that the unextracted nitrogen content was higher, the average being 0.43% (moisture-free basis).

As a result, in both these experiments a lower percentage of the total nitrogen present in the 500-mg. portion was extracted from barley samples of low total nitrogen content than from samples of high total nitrogen content. Table III shows the results obtained with a number of barley samples in these two experiments. The finding that greater amounts of barley proteins are brought into solution by alkaline copper sulfate solutions than by alkaline solutions alone is in accord with the work on corn proteins by Mertz and Bressani (6). However, the addition of sodium sulfite (6) had no effect on the amount of nitrogen extracted or the amount of biuret color produced under the conditions applied in the experiments reported in this paper.

The results (Table III) were plotted as three curves: the amounts of nitrogen actually extracted, the amounts of nitrogen actually present, and the percentage nitrogen content on a moisture-free basis in

TABLE III

UNEXTRACTABLE NITROGEN CONTENT AND PERCENTAGE OF TOTAL NITROGEN CONTENT EXTRACTED FROM BARLEY[a]

| Barley Nitrogen (mfb)[b] | Moisture | Nitrogen In 500 mg. | Nitrogen Extracted | Total Nitrogen Extracted | Nitrogen Not Extracted | Unextracted Nitrogen (mfb) | Absorbance of Biuret Extract |
|---|---|---|---|---|---|---|---|
| % | % | mg | mg | % | mg | % | |
| 1.16 | 10.9 | 5.17 | 4.28 | 82.7 | 0.89 | 0.20 | 0.441 |
| 1.53 | 11.0 | 6.81 | 6.10 | 89.6 | 0.71 | 0.16 | .500 |
| 1.82 | 11.0 | 8.10 | 7.08 | 87.4 | 1.02 | 0.23 | .520 |
| In absence of Cu tartrate | | | 6.35 | 78.2 | 1.75 | 0.40 | .107 |
| 1.86 | 11.2 | 8.26 | 7.35 | 89.0 | 0.91 | 0.20 | .562 |
| 2.10 | 10.7 | 9.38 | 8.93 | 95.2 | 0.45 | 0.10 | .628 |
| 2.16 | 11.0 | 9.62 | 8.87 | 92.3 | 0.75 | 0.17 | .599 |
| In absence of Cu tartrate | | | 7.93 | 82.5 | 1.69 | 0.38 | .117 |
| 2.47 | 11.6 | 10.92 | 10.35 | 94.9 | 0.57 | 0.13 | .653 |
| 2.51 | 11.2 | 11.14 | 10.58 | 95.0 | 0.56 | 0.13 | .652 |
| 2.51 | 10.6 | 11.22 | 9.82 | 87.6 | 1.40 | 0.31 | .654 |
| In absence of Cu tartrate | | | 9.03 | 80.5 | 2.19 | 0.49 | .111 |
| 2.53 | 10.3 | 11.35 | 10.63 | 93.7 | 0.72 | 0.16 | .680 |
| 2.74 | 10.5 | 12.26 | 11.13 | 90.8 | 1.13 | 0.25 | .710 |
| In absence of Cu tartrate | | | 10.20 | 83.2 | 2.06 | 0.46 | .129 |
| 3.02 | 10.6 | 13.50 | 12.42 | 92.6 | 1.08 | 0.22 | .759 |
| 3.22 | 10.5 | 14.41 | 13.59 | 94.3 | 0.82 | 0.18 | .782 |
| In absence of Cu tartrate | | | 12.47 | 86.5 | 1.94 | 0.44 | .121 |
| 3.63 | 10.8 | 16.18 | 15.63 | 96.6 | 0.55 | 0.12 | 0.868 |

[a] 500-mg. portions of barley were extracted by method 1.
[b] %N(mfb), percentage nitrogen content on moisture-free basis.

500-mg. portions of the same samples plotted against the absorbance of the colors produced in the biuret procedure. When the resultant plots were examined, the relative position of each sample to the other samples and to the relevant correlation curve was almost the same in each case. This shows that analytical results expressed on any of these three bases are directly comparable. The relevant correlation coefficient, the error of the determination of the nitrogen content by the colorimetric method, and the equation for the relation between nitrogen content and absorbance are given for each plot in Table IV.

*Moisture Variation.* As Table IV shows, the variation in moisture content (which is normally ignored in both methods) has no significant effect on the relation between absorbance and nitrogen content in the biuret method.

*Absorbance Coefficient of Extracted Proteins.* Variations in the amount of extraneous color produced, in the amounts of free amino acids and peptides present (which exhibit different spectral absorption characteristics and absorbance coefficients to proteins in the biuret reaction), or in the amounts present of such compounds as nucleic acids, are unlikely to account for the entire deviation from the theoretical correlation curve. It has been shown that different

TABLE IV[a]

RELATION BETWEEN ABSORBANCE (METHOD 1) AND EXTRACTED NITROGEN AND TOTAL NITROGEN CONTENTS OF BARLEY

| ABSORBANCE OF BIURET COLOR CORRELATED WITH: | CORR. COEFF. | ERROR OF COLORIMETRIC DETERMINATION OF NITROGEN[b] (± MG. N OR %N(MFB)) | | REGRESSION EQUATION[c] |
|---|---|---|---|---|
| | | *mg* | *%* | |
| N extracted, mg. | r = 0.994** | 0.353 | | mg. N = (25.80A − 6.832) |
| N present, mg. | r = 0.993**[e] | 0.371 | | mg. N = (25.61A − 5.882) |
| % N (mfb)[d] | r = 0.992**[e] | | 0.085 | % N(mfb) = (5.716A − 1.302) |

[a] All values used in calculating these results were derived from Table III.
[b] The error was determined by the standard linear regression method.
[c] The regression equation showing the relation between nitrogen content and the absorbance (A) in the calculated line of best fit.
[d] %N(mfb): percentage nitrogen content on moisture-free basis.
[e] The difference between these two correlation coefficients is not statistically significant.

types of proteins exhibit different absorbance coefficients in both the biuret reaction (2) and the method of Lowry *et al.* (4), and it is suggested that differences in protein type and structure in the various varieties of wheat and barley account largely for nonperfect correlation in both methods. The examination of correlation curves, each derived from samples of several barley varieties, showed that each variety, regardless of nitrogen content, tended to maintain the same relative position to the correlation curve. For example, the proteins from Prior, Bonus, and Triple-Awned Lemma barleys showed absorbance coefficients which were respectively lower than, higher than, and very close to the mean absorbance coefficient of all barley varieties.

## Acknowledgments

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# A METHOD FOR THE DETERMINATION OF RELATIVE AMOUNTS OF MALTED-WHEAT, FUNGAL (Aspergillus oryzae) AND BACTERIAL (Bacillus subtilis) ALPHA-AMYLASE IN MIXTURES, AND ITS APPLICATION TO MALTED WHEAT[1]

JAMES R. FLEMING, BYRON S. MILLER, AND JOHN A. JOHNSON

## ABSTRACT

A method to determine relative amounts of malted-wheat, fungal (*Aspergillus oryzae*), and bacterial (*Bacillus subtilis*) alpha-amylase activities in mixtures is presented. The method depends on differences in the thermostability of the three types of amylases and the use of simultaneous algebraic equations to calculate the percentages. Results of analyses of known mixtures of the three sources agree well with known composition.

The presence of fungal alpha-amylase in malted wheat was closely related to the observed development of molds. There was little bacterial alpha-amylase in any of the malted wheats. The evidence suggests that contribution of the fungus to the total alpha-amylase activity of malted wheat is due to the production of fungal alpha-amylase, and not to gibberellic acid which could stimulate the production of cereal alpha-amylase. Up to 15% of the amylolytic activity of moldy malted wheat was due to fungal alpha-amylase.

Infestation of cereals by microorganisms is almost universal, and the moist conditions prevalent during malting are optimal for development of the hydrophilic segment of microflora. Most prior studies have been concerned mainly with detecting (13) and controlling (4,9) microorganisms during malting and the effects of selected organisms on malt properties (11). Little information is available concerning the amounts of alpha-amylase contributed by fungi and bacteria. Knight (7) used the difference in the thermostability of fungal and cereal alpha-amylase to detect and estimate in other than SKB units the amount of fungal alpha-amylase which had been added to flours.

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No method which permits the detection of bacterial alpha-amylase in mixtures has been found in the literature.

Miller, Johnson, and Palmer (8) compared the thermostability of fungal, bacterial, and malted wheat flour alpha-amylase. Bacterial *(Bacillus subtilis)* alpha-amylase was the most thermostable, cereal alpha-amylase possessed intermediate thermostability, and fungal *(Aspergillus oryzae)* alpha-amylase was the least thermostable. The purpose of the present study has been to devise a procedure for the determination of relative amounts of cereal, fungal, and bacterial alpha-amylase in mixtures and to apply this method to malted wheat.

## Materials and Methods

The sources of alpha-amylase used in this investigation consisted of several experimentally produced wheat malts (3), a fungal preparation (*Aspergillus oryzae*),[2,3] and a bacterial preparation (*Bacillus subtilis*)[2]. A standard malted wheat essentially free of mold was prepared by steeping the grain in 0.05% formaldehyde solution during the last hour of the steep period before germination (4).

Malts of Redlan sorghum, Kanota oats, Kindred barley, an unnamed white corn, and a hard red winter wheat were prepared under comparable conditions (germinated at 62°F. for 4 days) in order that the thermostability of alpha-amylases of these cereals could be compared. The alpha-amylase activities of these five malted cereals were 90, 68, 149, 51, and 161 SKB units per g., respectively.

Alpha-amylase activity was determined by a modified Wohlgemuth procedure (method IA) of Hagberg (5). This procedure makes possible the rapid detection of small quantities of alpha-amylase and the expression of activities in terms of the familiar SKB units.

The procedure was as follows: Extracts of malted-wheat, fungal and bacterial alpha-amylases were diluted to 470 ml. with distilled water and 25 ml. of a sodium acetate-acetic acid buffer. The buffer was made by mixing 40 ml. of a 4.0 molar solution of glacial acetic acid with 460 ml. of a 4.0 molar solution of anhydrous sodium acetate. The final pH of the buffered enzyme solutions, which also contained 0.2% calcium chloride, was 5.55. The initial alpha-amylase activity of the mixtures ranged from 50 to 600 SKB units per 470 ml. of liquid. Fifty grams of flour were added to a duplicate series of the diluted extracts because of the known stabilizing effect of substrates

[2] Rhozyme-33 is a fungal and Rhozyme-39 a bacterial concentrate. (Rohm and Haas Co., Philadelphia, Pennsylvania.)

[3] The mention in this publication of a trade product, equipment, or a commercial company does not imply its endorsement by the U.S. Department of Agriculture over similar products or companies not named.

on certain enzymes (8). Both series of extracts were heated in an amylograph and aliquots were removed at 30° and at 5°C. intervals from 60° to 95°C.

Aliquots were immediately chilled in an ice-water bath, centrifuged when necessary, and analyzed for alpha-amylase.

## Results and Discussion

The data in Table I, representing the average of eight replicate

TABLE I
RELATIVE THERMOSTABILITY OF MOLD-FREE MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASES

| Temperature | Enzyme Retained in Presence of Flour | | | Enzyme Retained with No Flour Present | | |
|---|---|---|---|---|---|---|
| | Malted-Wheat | Fungal | Bacterial | Malted-Wheat | Fungal | Bacterial |
| °C | % | % | % | % | % | % |
| 65 | 100 | 100 | 100 | 100 | 100 | 100 |
| 70 | 100 | 54.0 | 100 | 100 | 53.6 | 100 |
| 75 | 58.0 | 5.0 | 100 | 60.0 | 3.7 | 100 |
| 80 | 28.0 | 1.6 | 100 | 26.0 | 1.0 | 94 |
| 85 | 2.1 | .. | 95.0 | 1.6 | .. | 60 |
| 90 | .. | .. | 74.0 | .. | .. | 21 |
| 95 | .. | .. | 18.0 | .. | .. | 7 |
| Std. Dev. | | | | | | |
| 65 | 0.8 | 0.4 | 0.8 | 1.0 | 0.4 | 0 |
| 70 | 0.2 | 2.5 | 0.8 | 1.8 | 2.6 | 2.5 |
| 75 | 3.1 | 0.2 | 1.0 | 2.6 | 0.5 | 2.0 |
| 80 | 3.4 | 0.1 | 0.4 | 2.8 | 1.3 | 1.7 |
| 85 | 0.4 | .. | 3.5 | 0.3 | .. | 3.6 |

experiments, indicate a pronounced difference in the stability of the various alpha-amylases to heat. Fungal alpha-amylase was the most thermolabile and the bacterial enzyme the most stable. The data agree with those reported previously (8) when this same procedure was employed. As also shown previously, the presence of flour had relatively little effect on the thermostability of wheat-malt and fungal amylases, but had a pronounced protective effect on the bacterial amylase at temperatures above 80°C. Adding flour to all enzyme extracts was adopted as a routine practice in this study.

Knight (7) reported a measurable decrease in wheat-malt alpha-amylase heated at 68°C. for 30 minutes. In the present work, there was no decrease at 70°C. This difference is probably due to the rapid heating (1.5°C. per minute) in the amylograph. It could be partly due to the presence of fungal amylase in the malt used by Knight (7). In the present work, fungi were eliminated by the use of a formaldehyde treatment (4).

The data in Table I suggest that it should be possible to determine the relative amounts of each enzyme by the use of simultaneous equations. The percentages of each type of alpha-amylase remaining after heating at three temperatures are used as coefficients. The activity of all three alpha-amylases was 100% at 65°C., thus:

$$C + F + B = \text{total activity}$$

where C represents the contribution of cereal (wheat-malt) alpha-amylase, F that of fungal alpha-amylase, and B that of bacterial alpha-amylase to the total activity. At 70°C. only 54% of the fungal alpha-amylase is retained, thus:

$$C + 0.54\,F + B = \text{total activity}$$

Similarly, at 75°C., 58% of the cereal and 5% of the fungal alpha-amylase are retained, thus:

$$0.58\,C + 0.05\,F + B = \text{total activity}$$

The amount of each type of alpha-amylase present in a given enzyme mixture may be determined by solving for the unknowns in the three simultaneous equations. Since the thermostability of malted-wheat alpha-amylase may be somewhat different from that of other cereals, thermostability data for the amylase in these cereals should be obtained before attempting to apply this method.

The proposed method was tested by analyzing mixtures of the three enzymes. The amount of each component varied from 5 to 90% of the total. The data given in Table II, which were obtained by

TABLE II

MEASURED AND CALCULATED AMOUNTS[a] OF MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASE IN KNOWN MIXTURES

| Total Activity | Mold-Free Malted-Wheat | | Fungal | | Bacterial | |
|---|---|---|---|---|---|---|
| | Added | Found | Added | Found | Added | Found |
| *SKB units* | % | % | % | % | % | % |
| 156 | 80.0 | 81.8 | 15.0 | 15.4 | 5.0 | 2.8 |
| 146 | 80.0 | 78.8 | 10.0 | 10.4 | 10.0 | 10.8 |
| 155 | 75.0 | 65.8 | 5.0 | 4.2 | 25.0 | 30.0 |
| 153 | 60.0 | 56.5 | 20.0 | 21.3 | 20.0 | 22.2 |
| 150 | 50.0 | 49.0 | 30.0 | 30.6 | 20.0 | 20.4 |
| 156 | 50.0 | 47.7 | 20.0 | 20.9 | 30.0 | 37.4 |
| 150 | 33.3 | 31.5 | 33.3 | 33.3 | 33.3 | 35.2 |
| 156 | 30.0 | 28.5 | 30.0 | 30.8 | 40.0 | 40.7 |
| 156 | 20.0 | 23.7 | 40.0 | 40.4 | 40.0 | 35.9 |
| 150 | 10.0 | 12.0 | 45.0 | 43.5 | 45.0 | 44.5 |
| 146 | 5.0 | 9.5 | 45.0 | 41.8 | 50.0 | 48.7 |
| 154 | 10.0 | 9.2 | 80.0 | 80.5 | 10.0 | 10.3 |
| 146 | 20.0 | 20.8 | 60.0 | 58.2 | 20.0 | 21.0 |
| 150 | 10.0 | 10.0 | 10.0 | 10.1 | 80.0 | 79.9 |
| 153 | 20.0 | 20.9 | 20.0 | 19.6 | 60.0 | 59.5 |

[a] Percent of total alpha-amylase activity.

TABLE III

RELATIVE AMOUNTS OF MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASE IN MALTS DIFFERING IN THEIR CONTAMINATION WITH MOLD

| Moldiness of Malt Sample | Total Alpha-Amylase Activity | | | Sources of Alpha-Amylase Activity | | |
|---|---|---|---|---|---|---|
| | At 65°C. | At 70°C. | At 75°C. | Malted-Wheat | Fungal | Bacterial |
| | *SKB units/g* | *SKB units/g* | *SKB units/g* | %[a] | %[a] | %[a] |
| Slight | 140.0 | 138.4 | 79.2 | 97.2 | 2.5 | 0.3 |
| Slight | 148.0 | 145.5 | 83.0 | 96.0 | 3.7 | 0.3 |
| Slight | 145.0 | 140.3 | 78.7 | 92.2 | 7.1 | 0 |
| Moderate | 145.0 | 140.8 | 79.7 | 93.1 | 6.3 | 0.6 |
| Heavy | 139.7 | 133.0 | 73.3 | 89.5 | 10.4 | 0.1 |
| Heavy | 152.7 | 145.0 | 79.8 | 88.7 | 10.9 | 0.4 |
| Very heavy | 163.3 | 152.2 | 82.2 | 84.9 | 14.8 | 0.3 |

[a] Percent of total activity.

more than one operator at different times, show the distribution of the three sources of alpha-amylase in the mixtures. The amount of alpha-amylase "found" in each mixture in most instances agreed with the "known" amount within acceptable experimental limits. Since the activity of fungal amylase decreases very rapidly between 65° and 75°C. and that of malt amylase between 75° and 85°C., samples must be withdrawn and cooled rapidly according to the same fixed experimental procedure for both known and unknown sample mixtures.

The data in Table III show the relative amounts of cereal, fungal, and bacterial alpha-amylase in a series of malted wheats which differed in apparent moldiness. The most heavily infested samples were produced deliberately by introducing a small quantity of moldy wheat at the start of the germination period. These malts were comparable in moldiness to some other samples produced in a prior investigation (3). In general, the malts which appeared to be the least contaminated by mold contained the least fungal amylase. The amount of bacterial alpha-amylase found was slight in all instances. This finding agrees with observations made during an earlier study (4) when malts were tested for microorganisms by use of sterile malt agar plates.

The relative amounts of fungal alpha-amylase found in malted wheats (Table III), particularly those in which mold growth was moderate to heavy, agree with data by Prentice and Sloey (11) which were obtained after intentionally adding fungi during malting. While Sheneman and Hollenbeck (13) found appreciable numbers of bacteria present during malting of barley, Prentice and Sloey (11) found that bacteria added deliberately during the malting process caused no significant increase in alpha-amylase activity of finished malt.

The present data confirm those of Prentice and Sloey (11) and Sheneman and Hollenbeck (13).

Prentice and Sloey (11) suggested that the increase in total alpha-amylase activity of cereal malts in which there was an associated development of mold might be due either to the action of fungus-produced gibberellins or to the production of fungal alpha-amylase. Fleming and Johnson (2) have shown that growth of beta-amylase and acrospires increases in malt under the influence of gibberellins. The lack of 1) a significant increase in beta-amylase activity and 2) a stimulating effect on acrospire growth obtained by Prentice and Sloey (11) militates for the possibility that the increased alpha-amylase and protease activities could be due to stimulation by fungus-produced gibberellins. It was established in the present study that up to 15% of the alpha-amylase in moldy malted wheat was of fungal origin.

The coefficients in the equations used for measuring the relative amounts of cereal, fungal, and bacterial alpha-amylase may be strictly valid only for malted wheat and the specific *Aspergillus oryzae* and *Bacillus subtilis* preparations studied. Similar data, however, can be obtained for other sources of the enzyme. Conn, Johnson, and Miller (1) demonstrated that the thermostability was not the same for different sources of bacterial amylase, but no data are available for different sources of fungal amylase. Data concerning the thermostability of different cereal alpha-amylases are given in Table IV. They represent triplicate analyses. All of the cereal alpha-amylases, with the exception of that from oats, were stable at temperatures up to 70°C. under the established conditions. Sorghum and barley

TABLE IV

RELATIVE THERMOSTABILITY OF MALTED-CEREAL ALPHA-AMYLASES

| Temperature | Enzyme Activity Retained in the Presence of Flour | | | | |
|---|---|---|---|---|---|
| | Wheat | Sorghum | Oats | Barley | Corn |
| °C | % | % | % | % | % |
| 60 | 100 | 100 | 100 | 100 | 100 |
| 65 | 100 | 100 | 98.4 | 100 | 100 |
| 70 | 100 | 100 | 92.2 | 100 | 100 |
| 75 | 58.0 | 63.6 | 53.9 | 61.3 | 59.0 |
| 80 | 28.0 | 33.6 | 21.5 | 33.8 | 27.8 |
| 85 | 2.1 | 4.0 | 3.1 | 4.4 | 2.7 |
| Std. Dev. | | | | | |
| 60 | 0 | 0 | 0 | 0 | 0 |
| 65 | 0.8 | 1.3 | 0.4 | 0 | 0 |
| 70 | 0.2 | 1.3 | 1.9 | 0 | 0 |
| 75 | 3.1 | 2.1 | 0.5 | 1.6 | 3.0 |
| 80 | 3.4 | 0.4 | 0.8 | 0.6 | 4.0 |
| 85 | 0.4 | 0.4 | 0.1 | 2.1 | 1.3 |

alpha-amylases were slightly more stable than those of wheat and corn at 75°C.; that of oats was less stable. The same trend was observed at 80°C. These data do not agree with those of Kneen (6), who reported that sorghum alpha-amylase was less stable than that of barley.

Pronin (12), contrary to the present work, indicated that wheat-malt alpha-amylase was more stable then barley alpha-amylase. This could be due to differences in malting procedure, heat-inactivation procedure, or varietal response. Kneen (6) found a difference in the stability of alpha-amylase of sorghum malts produced from different varieties. The data in Table V pertaining to two varieties of hard red winter wheat (Triumph and Bison), and one variety each of hard red spring (Rescue), durum (Cernum), and white winter wheat (Brevor) indicate that varietal differences, though slight, might be enough to account, in part, for the differences obtained by different workers. The problem of comparing stabilities of alpha-amylase from malts of varying origins may be complicated by the effect of kilning conditions. Preece (10) found that high kilning temperatures increased the sensitivity of heat of malted-barley alpha-amylase. Differences in other phases of the malting procedure may also explain part of the differences obtained in different laboratories. In the present investigation the heating was uniform at a fixed rate in an amylograph. Other means of heating were likely employed by the other workers (6,12).

The present data and those of others (6,10,12) serve to emphasize the necessity for strict control of many variables in order to apply the proposed method of analysis. After suitable preliminary investigations based on the principle of the present method, the technique can be applied to many specific problems. A general application might be for measuring the amount of fungal amylase in flour where such supplementation is permitted.

TABLE V

EFFECT OF WHEAT VARIETY ON THE THERMOSTABILITY OF MALTED WHEAT ALPHA-AMYLASE

| Temperature | Wheat Variety | | | | |
|---|---|---|---|---|---|
| | Triumph | Bison | Rescue | Vernum | Brevor |
| °C | %[a] | %[a] | %[a] | %[a] | %[a] |
| 65 | 100 | 100 | 100 | 100 | 100 |
| 70 | 100 | 100 | 99.3 | 100 | 98.8 |
| 75 | 58.1 | 60.0 | 58.1 | 60.0 | 56.2 |
| 80 | 28.1 | 29.9 | 26.9 | 29.4 | 25.7 |

[a] Percent of original alpha-amylase activity.

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# CHLORIDE CONTENT OF CAKE FLOURS AND FLOUR FRACTIONS[1]

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## ABSTRACT

Unbleached cake flours contained 43 to 54 mg. chloride per 100 g. flour. Over 60% of this chloride appeared in the water-solubles. The other fractions were very low in chloride content. However, the prime starch, which often amounted to over 70% of the flour in these low-protein cake flours, contained 20 to 25% of the flour chloride.

Chlorine-bleached cake flours contained 131 to 189 mg. chloride per 100 g. flour. Nearly all this increased chloride was in and rather equally divided between the water-solubles and the gluten. The tailings contained a small part of the increased chloride, and the prime starch had very little of the increase. The chloride of the bleached water-solubles appeared almost entirely in the low-molecular-weight part. More than half of the chloride of the bleached gluten was held in the lipids, but the protein residue retained substantial amounts.

In a cake flour treated with chlorine at five different levels, the increased chloride appeared chiefly in and about equally divided at all levels between the water-solubles and the gluten. The tailings gained chloride slowly. The prime starch did not show any consistent increase.

An improvement in an acetic acid fractionation procedure for flour was made by recovering the soluble protein from the supernatant by dialysis and lyophilization. This made possible 98 to 99% recoveries of dry matter and protein.

Today practically all cake flours are bleached with chlorine or some blend of gases in which chlorine is the principal constituent, but very little is known of the chemistry involved. Earlier work at this laboratory (16) had shown that bleached prime starch was responsible for most of the improvement from bleaching, but that bleached gluten accounted for some.

The determination of the chloride content of bleached prime starch compared to that of unbleached prime starch was the first objective of the present study. After preliminary work showed that bleached prime starch had a very low chloride content, the study was extended to the other fractions. About 150 to 200 mg. of chlorine are required to bleach 100 g. of flour to a pH of 4.8, and the fate of that chlorine after bleaching is the subject of this paper.

Several earlier workers determined the chloride content of flours. Bailey (2) has reviewed the earlier work. Utt (21), in 1914, obtained

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samples of the same flours before and after chlorine bleaching in a Kansas flour mill. He found chloride contents ranging from 0.044 to 0.58% – this is equivalent to 44 to 58 mg. chloride per 100 g. flour – for unbleached flours and from 0.065 to 0.097% for bleached flours. These flours were evidently intended for bread-baking, and the level of chlorine treatment probably was lower than that used today for cake flours, since one authority (9) quoted levels of 1 to 2 oz. per 280 lb. for bread flours and 4 to 5 oz. for cake flours. Sullivan and Howe (19) reported 0.051% for an unbleached patent flour from a hard spring wheat. Pap (12) found that 32 Hungarian flours ranged from 0.060 to 0.074%; it is not clear whether all these flours were unbleached. Damiens and Blaignan (5) obtained 43 to 77 mg. chloride per 100 g. flour.

## Materials and Methods

*Flours.* Both commercial and laboratory-milled cake flours were used in this study. The commercial cake flours were included in each experiment. The first lot of commercial cake flour, from the 1957 crop, became depleted during this study; another lot, from the 1959 crop, was obtained. Both lots were milled from western soft wheats. Each lot contained an unbleached flour and a flour bleached at the mill with chlorine to pH 4.8.

The laboratory flours were prepared by combining the first two break streams and the first two reduction streams from a Buhler mill and passing the composite through a 200-mesh stainless-steel screen. One laboratory flour was milled from Brevor wheat, a soft, white common variety; and the other was from Elmar, a club wheat. Both varieties have good cake-baking quality. These flours were similar in particle size distribution to the commercial cake flour and yielded layer cakes of similar volume and texture.

*Flour Bleaching.* A portion (400 g.) of each laboratory-milled flour was treated in a laboratory bleacher at room temperature (72°F.) with chlorine gas of 99.5% purity. The flour was placed in the bleacher, the bleacher was evacuated to about 400 mm. mercury, the chlorine was introduced over a period of 1 minute with agitation, and agitation was continued for 4 minutes. Before the desired flour was treated, several preliminary batches were bleached and discarded; this was to remove air from most of the apparatus. The results obtained were:

| *Flour* | *Before Bleaching* | | | *Chlorine* | *After Bleaching* | | |
|---|---|---|---|---|---|---|---|
| | *Moisture* | *Protein* | *pH* | | *Immediate pH* | *Storage Time* | *pH at Fractionation* |
| | % | % | | mg/100 g flour | | days | |
| Brevor | 12.1 | 6.9 | 5.82 | 200 | 4.92 | 28 | 4.84 |
| Elmar | 12.3 | 7.4 | 5.84 | 220 | 4.83 | 5 | 4.81 |

Portions (500 g.) of the 1959 unbleached commercial cake flour were bleached at five different levels in the laboratory bleacher. This flour had a moisture content of 12.6%, a protein content of 6.87% at 12.6% moisture, and an initial pH of 5.91. The results obtained were:

| *Chlorine* | *pH Immediately after Bleaching* | *At Fractionation* | |
|---|---|---|---|
| | | *pH* | *after Bleaching* |
| *mg/100 g flour* | | | *days* |
| 48 | 5.58 | 5.50 | 2 |
| 96 | 5.24 | 5.22 | 6 |
| 144 | 5.05 | 4.90 | 8 |
| 192 | 4.79 | 4.41 | 13 |
| 240 | 4.40 | 4.22 | 15 |

About 190 mg. of chlorine per 100 g. flour would have given a pH of 4.8 at the time of bleaching, and about 154 mg. of chlorine would have resulted in a pH of 4.8 after 1 or more weeks of storage.

*Flour Fractionation.* All fractionations but one were made by a modification of the acetic acid extraction procedures (14,15). Concentration of the water-extract was omitted, and a dry product was obtained by shelling and lyophilizing the water-extract. The suspension of bleached flour and distilled water was adjusted to pH 6.0 (15). The gluten that precipitated was obtained, and, in addition, the soluble protein remaining in the supernatant, after neutralization and centrifugation, was recovered.

The recovery of soluble protein was made by dialysis followed by lyophilization. The unconcentrated supernatant (pH about 6.0) was dialyzed against distilled water with changes of water at 2-hour intervals four times each day for 2 days. In one experiment, a large volume of supernatant was divided into four equal portions, and portions were dialyzed for two, four, six, and eight changes of water. The soluble protein was recovered and analyzed. The results were:

| *Times Water Changed* | *Weight Recovered* | *Percent Protein* | *Amount Protein Recovered* |
|---|---|---|---|
| | *g* | *%* | *g* |
| 2 | 0.66 | 34.7 | 0.23 |
| 4 | 0.52 | 43.8 | 0.23 |
| 6 | 0.46 | 52.3 | 0.24 |
| 8 | 0.41 | 62.0 | 0.25 |

The two fractions containing most of the chloride were further subdivided. The water-solubles were separated into the alcohol-solubles (low-molecular-weight materials as sugars and amino acids) and the alcohol-insolubles (water-soluble proteins and polysaccharides)

(17). The water-extracts from several flours were prepared, and each was divided into two equal parts. One part was poured into three volumes of ethanol (17). The alcohol-insolubles were removed by centrifuging and redissolved in water. Aliquots of these solutions and of the supernatants (alcohol-soluble material) were analyzed for dry matter and chloride. From these two values the percent chloride was calculated. Aliquots of the original water-extract were likewise analyzed.

The glutens were separated into the lipid portion and the protein residue by extracting with water-saturated normal butanol (11). Aliquots of the entire butanol extract (crude lipids) were taken, and the butanol evaporated on the steam bath. Chloride was determined on the crude lipids. For dry matter, the lipids and the protein residue were dried at 100°C. under vacuum for 4 hours.

One fractionation was made by the doughing procedure developed by Udy (20) so that fractions from a separation in which acid was not used might be compared with the fractions from the acetic acid separations. This was with the unbleached cake flour; all attempts to fractionate bleached cake flour by doughing were unsuccessful.

*Analytical Methods.* Moisture, protein, and pH were determined by conventional methods (1,10). Particle size measurements were made by a sedimentation method (15).

Determination of total chloride on a macro scale used ashing for digestion. Samples of 5 g. for flours, prime starches, and tailings and 1 g. for water-solubles and glutens were slurried with 25 ml. of 2% potassium carbonate to prevent loss of chloride (8,19,21), dried on the steam bath, and ashed for 2 hours at 550°C. (8). The ashed samples were transferred to beakers with 5 ml. of 1.5*N* nitric acid and 50 ml. distilled water, and a conventional gravimetric determination was used (13). Final precipitates ranging from 10 to 50 mg. of silver chloride were obtained except with starches, tailings, and unbleached glutens.

Determination of total chloride on a micro scale required 1 to 100$\gamma$ of chloride. These quantities were obtained through either ashing, Carius digestions, or Schöniger combustions. For ashing, samples of 100 mg. were slurried with 6 ml. of 2% potassium carbonate, dried, and ashed for 2 hours at 550°C. (8). The residues were transferred with 5 ml. 1.5*N* nitric acid and distilled water to either beakers or 50-ml. volumetric flasks. Carius digestions were made by a conventional method (18). Samples of 50 mg. plus 0.5 ml. fuming nitric acid were sealed in glass tubes and heated inside water-pipe bombs for 2 hours at 250°C. The residues were transferred with distilled water to

beakers or volumetric flasks. Schöniger combustions were carried out in a commercial apparatus[3]. Samples of 50 mg. were burned in oxygen and transferred to beakers or volumetric flasks.

Some of the methods available for determination of chloride on a micro scale have been reviewed by Gunther and Blinn (6). Potentiometric determination followed closely the method of Helmkamp *et al.* (6,7). Best results were obtained when the silver electrode was cleaned and recoated each day with silver chloride. Measurements of mv. were made with a student potentiometer[4] and a sensitive galvanometer. The colorimetric determination was that of Bergmann and Sanik (3) adapted to a final volume of 59 ml.

## Results

*Flour Fractionation.* The earlier acetic acid extraction procedures for flour fractionation (14,15) gave 97 to 99% recoveries of dry materials but only 83 to 87% recoveries of flour protein for cookie flours and as low as 79% protein recovery for a bleached cake flour. In the present study, the recovery of the protein remaining soluble after neutralization substantially increased the recovery of flour protein, as shown in Table I; 98 to 99% of the protein can be recovered by careful work. Table I also shows the yields and protein contents of the fractions and the protein contents of the original flours.

TABLE I
YIELDS AND PROTEIN CONTENTS OF CAKE FLOURS AND FRACTIONS

| Material | 1959 Commercial Unbleached Flour | | 1959 Commercial Bleached Flour | | 1959 Commercial Unbleached Flour: Doughing Procedure | |
|---|---|---|---|---|---|---|
| | Yield[a] | Protein[b] | Yield | Protein | Yield | Protein |
| | g | % | g | % | g | % |
| Flour | 100.0 | 6.76 | 100.0 | 6.59 | 100.0 | 6.76 |
| Fractions | | | | | | |
| Water-solubles | 4.1 | 17.7 | 3.9 | 15.4 | 4.5 | 18.5 |
| Gluten | 6.7 | 69.2 | 6.4 | 65.2 | 5.6 | 64.6 |
| Soluble protein | 1.3 | 55.9 | 1.8 | 56.4 | .... | .... |
| Tailings starch | 13.2 | 3.01 | 15.0 | 3.08 | 21.8 | 9.15 |
| Prime starch | 74.3 | 0.30 | 72.6 | 0.32 | 67.4 | 0.28 |
| Dry matter recovery, % | 99.6 | .... | 99.7 | .... | 99.3 | .... |
| Protein recovery, % | .... | 99.5 | .... | 98.6 | .... | 99.1 |

[a] Grams at 14% moisture from 100.0 g. flour at 14% moisture.
[b] At 14% moisture.

[3] Arthur H. Thomas Co., Philadelphia, Pa. Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
[4] No. 83411, Central Scientific Co., Chicago, Ill.

TABLE II

CHLORIDE CONTENTS OBTAINED BY DIFFERENT ANALYTICAL METHODS FOR A BLEACHED CAKE FLOUR AND ITS FRACTIONS

| MATERIAL | ASHING PLUS GRAVIMETRIC | | ASHING PLUS POTENTIOMETRIC | | COMBUSTION PLUS COLORIMETRIC | | COMBUSTION PLUS POTENTIOMETRIC | |
|---|---|---|---|---|---|---|---|---|
| | Chloride Content[a] | Std. Error | Chloride Content | Std. Error | Chloride Content | Std. Error | Chloride Content | Std. Error |
| | mg | mg | mg | mg | mg | mg | mg | mg |
| Flour[b] | 125 | 2 | 137 | 1 | 149 | 9 | 89 | 6 |
| Fractions | | | | | | | | |
| Water-solubles | 1,490 | 14 | 1,480 | 35 | 1,350 | 36 | 1,090 | 14 |
| Gluten | 499 | 3 | 533 | 13 | 520 | 24 | 407 | 7 |
| Soluble protein | 272 | 14 | 297 | 9 | 297 | 21 | 194 | 7 |
| Tailings starch | 35 | 4 | 39 | 2 | 43 | 6 | 36 | 4 |
| Prime starch | 7 | 2 | 18 | 4 | 10 | 4 | 6 | 1 |
| Chloride recovery, % | 84 | .. | 85 | .. | 71 | .. | 91 | .. |

[a] Chloride in mg. per 100 g. of the fraction or flour indicated; all results are on a 14% moisture basis.
[b] A 1957 commercial bleached cake flour.

*Chloride Analysis.* Table II lists the chloride values obtained by the useful combinations of digestions and determinations for a commercial bleached cake flour and fractions. Three combinations not included in Table II gave high and erratic values for blanks, standards, and samples: ashing followed by colorimetric determination, and Carius digestions with either colorimetric or potentiometric determinations. The gravimetric determination was not used with the digests from the Carius digestion and the Schöniger combustions because of the small sample size. Bethge and Troëng (4) have reported successful combustions of 500 mg. and more by using repeated ignitions with the same absorption liquid.

Ashing plus gravimetric determination gave good recoveries of standards and reproducible values for samples but was tedious and time-consuming. Schöniger combustions followed by colorimetric or potentiometric determinations gave low results, as shown in Table II, although Bethge and Troëng (4) reported higher recoveries for chloride in wood pulp by combustion than by ashing.

Ashing combined with potentiometric determinations gave reproducible values for standards and values for samples that were equal to or slightly higher than results by ashing plus gravimetric determination. Ashing plus potentiometric determination was reliable, was adaptable to large numbers of samples, was tolerant to a large range in sample size, and was rapid. It was adopted for all further analyses in this study.

*Recovery of Chloride in Fractions.* Recoveries of chloride in the

fractions ranged from 80 to 100% and averaged 89% for the 14 fractionations in which the chloride content of all the fractions was determined by ashing combined with the potentiometric method.

The recoveries of chloride in the fractions were rather low. Possibilities considered in trying to account for the low recoveries were:

1. Recovery of dry matter averaged 99%; an adjustment for this 1% loss would raise chloride recovery about 1% and more if part of a fraction high in chloride were lost. One known loss was that of water-solubles which were lyophilized in large glass bottles. A small amount was always left in the bottle. In several instances this residue was dissolved in water, and aliquots were analyzed. Amounts of chloride were found that would raise the recovery about 1% from this source alone.

2. Gluten samples were more difficult to determine than the other fractions, therefore:
   a. Gluten chloride values may be somewhat low.
   b. Losses of chloride may have occurred during dialysis of the soluble protein.

*Chloride Content of Flours and Fractions.* The chloride values for three pairs of unbleached and bleached cake flours and their fractions are shown in Table III.

Unbleached flour had an appreciable chloride content. During growth the wheat plant evidently deposits chloride from the soil in the endosperm. The three flours listed ranged from 43 to 54 mg. per 100 g. flour. This is in good agreement with Utt (21) and Sullivan and Howe (19) and is somewhat lower than Pap (12) and Damiens and Blaignan (5). Of the fractions from unbleached flours, the water-solubles were high in chloride content, ranging from 630 to 852 mg.

TABLE III

Chloride Contents of Unbleached and Bleached Cake Flours and Fractions

| Material | Chloride Content [a] | | | | | |
|---|---|---|---|---|---|---|
| | 1959 Commercial Flour | | Brevor Flour | | Elmar Flour | |
| | Unbleached | Bleached | Unbleached | Bleached | Unbleached | Bleached |
| | mg | mg | mg | mg | mg | mg |
| Flour | 54 | 131 | 43 | 145 | 46 | 189 |
| Fractions | | | | | | |
| Water-solubles | 852 | 1,690 | 630 | 1,300 | 751 | 1,840 |
| Gluten | 17 | 463 | 19 | 666 | 10 | 937 |
| Soluble protein | 63 | 271 | 27 | 538 | 62 | 426 |
| Tailings starch | 16 | 49 | 18 | 42 | 13 | 52 |
| Prime starch | 13 | 15 | 17 | 20 | 13 | 18 |

[a] Chloride in mg. per 100 g. of the fraction or flour indicated; all results are on a 14% moisture basis.

TABLE IV
DISTRIBUTION AMONG FRACTIONS OF THE CHLORIDE FROM ONE HUNDRED GRAMS OF CAKE FLOUR

| Material | Amount of Chloride[a] | | | | | |
|---|---|---|---|---|---|---|
| | 1959 Commercial Flour | | Brevor Flour | | Elmar Flour | |
| | Unbleached | Bleached | Unbleached | Bleached | Unbleached | Bleached |
| | mg | mg | mg | mg | mg | mg |
| Flour | 54 | 131 | 43 | 145 | 46 | 189 |
| Fractions | | | | | | |
| Water-solubles | 34.9 | 65.9 | 26.1 | 59.9 | 29.6 | 76.7 |
| Gluten | 1.1 | 29.6 | 1.4 | 42.6 | 0.7 | 65.4 |
| Soluble protein | 0.8 | 5.0 | 0.3 | 7.5 | 0.7 | 5.1 |
| Tailings starch | 2.1 | 7.4 | 3.7 | 7.2 | 1.9 | 11.3 |
| Prime starch | 9.7 | 11.6 | 11.2 | 13.5 | 9.4 | 11.8 |
| Total | 48.6 | 119.5 | 42.7 | 130.7 | 42.3 | 170.3 |
| Chloride recovery, % | 90 | 91 | 99 | 90 | 92 | 90 |

[a] Amount of the flour chloride found in the fraction indicated (yield of fraction from 100 g. flour times chloride content of the fraction).

per 100 g. water-solubles, while the other fractions were all very low in chloride content.

The bleached flours studied ranged from 131 to 189 mg. chloride per 100 g. flour. This was two and one-half to four times as high as the unbleached flours. The water-solubles from bleached flours had about twice the chloride content of those from unbleached flours, whereas the bleached glutens had much greater chloride contents than the unbleached glutens. Bleached tailings were three to four times as high in chloride as those from unbleached flours, but the prime starches increased only very slightly in chloride after bleaching.

*Distribution of Flour Chloride among Fractions.* The water-solubles and the gluten plus the soluble protein were rather small parts by weight of the flour – about 4 and 8 g., respectively, from 100 g. of the cake flours studied. In contrast, the prime starch usually amounted to over 70 g. from 100 g. of cake flour. The distribution of the flour chloride among the fractions was calculated and is shown in Table IV.

Most of the chloride of the unbleached flours was in the water-solubles, although the relatively large weight of prime starch caused it to contain 20 to 25% of the chloride. The combined gluten plus soluble protein and the tailings starches contained very little chloride.

The increased chloride in the bleached flours was divided chiefly between the water-solubles and the combined gluten plus soluble protein. In the commercial flour, each of these fractions gained about 30 mg. or about 40% of the 77 mg. increase over the unbleached flour. In the laboratory-bleached flours, the water-solubles gained

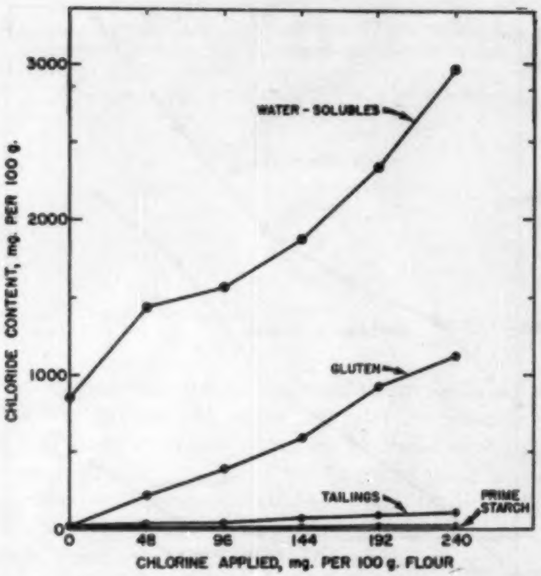


Fig. 1. Chloride contents of flour fractions at several levels of chlorine bleaching. The soluble protein is omitted to improve the legibility.

about 33% and the combined gluten plus soluble protein had about 40 to 45% of the increased chloride. The tailings accounted for 4 to 7% of the increase, and the prime starches held 2 to 3%.

*Results with Fractions Obtained by Doughing.* Table V compares the results of similar analyses of the fractions from a doughing procedure (20) with those of the fractions from an acetic acid separation. The results were about the same.

*Fractions from Flours Bleached with Varying Amounts of Chlorine.* Part of each of the flours bleached at five different levels in the laboratory was fractionated. The chloride content of the flours and their fractions was determined. The results are shown in Figs. 1 and 2. The soluble protein results were omitted from Fig. 1 for greater clarity; they did not differ greatly from the gluten results.

Figure 1 shows that the chloride content of the water-solubles, which was high in the beginning compared to the other fractions, increased as the amount of the chlorine applied to the flour increased and was consistently the highest of any of the fractions. The chloride content of the gluten increased with increasing amounts of applied

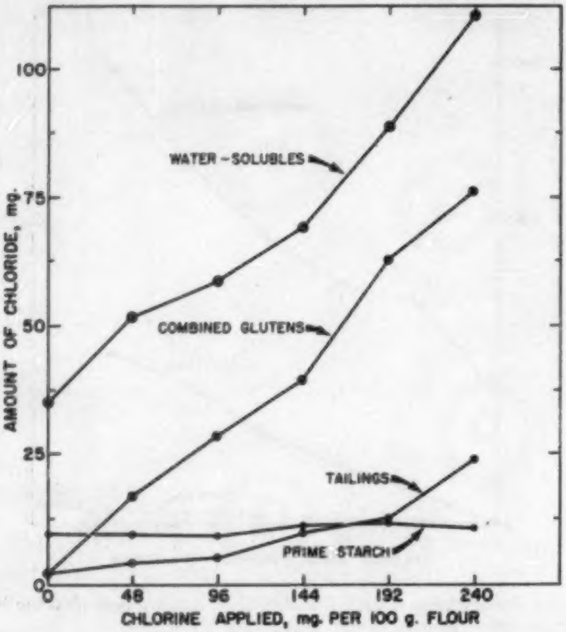


Fig. 2. Total amount of chloride in each fraction at several different levels of bleaching.

TABLE V

COMPARISON OF CHLORIDE CONTENTS OF, AND DISTRIBUTION OF, THE FLOUR CHLORIDE AMONG THE FRACTIONS OBTAINED FROM AN UNBLEACHED COMMERCIAL CAKE FLOUR BY AN ACETIC ACID EXTRACTION PROCEDURE AND BY A DOUGHING PROCEDURE

| MATERIAL | Type of Fractionation | | | |
|---|---|---|---|---|
| | Acetic Acid Fractionation | | Doughing Procedure | |
| | Chloride Content[a] | Amount of Chloride[b] | Chloride Content | Amount of Chloride |
| | *mg* | *mg* | *mg* | *mg* |
| Flour | 54 | 54 | 54 | 54 |
| Fractions | | | | |
| Water-solubles | 852 | 34.9 | 851 | 38.3 |
| Gluten | 17 | 1.1 | 12 | 0.7 |
| Soluble protein | 63 | 0.8 | ... | ... |
| Tailings starch | 16 | 2.1 | 9 | 2.0 |
| Prime starch | 13 | 9.7 | 9 | 6.1 |
| Total | ... | 48.6 | ... | 47.1 |
| Chloride recovery, % | ... | 90 | ... | 87 |

[a] Chloride in mg. per 100 g. of the fraction or flour indicated; all results are on a 14% moisture basis.
[b] Amount of flour chloride found in the fraction indicated (yield of fraction from 100 g. flour times chloride content of fraction).

TABLE VI

AMOUNT OF CHLORINE APPLIED, AMOUNT TAKEN UP BY THE FLOUR, AND PERCENT OF CHLORINE UTILIZED IN LABORATORY BLEACHING OF FLOURS

| Flour | Chlorine Applied[a] | Increase in Flour Chloride[a] | Chlorine Utilized |
|---|---|---|---|
| | mg | mg | % |
| Brevor | 200 | 102 | 51 |
| Elmar | 220 | 143 | 65 |
| Commercial | 48 | 41 | 86 |
| | 96 | 69 | 72 |
| | 144 | 88 | 61 |
| | 192 | 142 | 74 |
| | 240 | 190 | 79 |

[a] In mg. per 100 g. flour.

chlorine and reached high levels compared to the unbleached gluten. The tailings fraction showed slight increases in chloride, and the prime starch did not show any consistent increase in chloride.

Again, the difference in yield of the fractions must be considered to determine the total amount of chloride taken up by each fraction. This is done in Fig. 2, which shows that after the higher yield of gluten is considered, the two fractions, the water-solubles and the combined gluten plus soluble protein, each took up about the same amount of chlorine at any stage of bleaching.

Figure 2 also shows that there was no over-all increase in the amount of chlorine taken up by the prime starch. This differs somewhat from the data given in Tables III and IV which indicated slight increases in chloride for bleached prime starches.

*Chlorine Utilization by Flour in Laboratory Bleaching.* The differences between the amount of chlorine used in treating flour and the amount retained by the flour are illustrated in Table VI. At normal levels of bleaching, only one-half to two-thirds of the chlorine appeared in the flour.

The evidence from the commercial flour bleached at five different levels indicated that: 1) at low levels of chlorine treatment, nearly all the chlorine was taken up by the flour; 2) at normal levels of bleaching, a low point in the amount of chlorine taken up was reached; and 3) at higher than normal levels of bleaching, the utilization of chlorine improved markedly. The upswing in chloride contents and amounts shown for the water-solubles and glutens in Figs. 1 and 2 is explained by the higher utilization at these levels, since in these figures the contents and amounts were plotted against the amount of chlorine applied. If the chloride contents and amounts of the fractions were plotted against the amounts of chlorine retained

TABLE VII

CHLORIDE DISTRIBUTION AMONG THE SUBFRACTIONS OF THE WATER-SOLUBLE FRACTIONS OF SOME CAKE FLOURS

| Flour, Treatment, and Fraction | Yield[a] | Total Chloride | Chloride Content[b] |
|---|---|---|---|
| | g | mg | mg |
| 1959 Commercial unbleached: | | | |
| Total water-solubles: | 4.27 | 34.8 | 815 |
| Subfractions: | | | |
| Alcohol-soluble | 3.08 | 31.5 | 1020 |
| Alcohol-insoluble | 1.03 | 0.8 | 78 |
| Total | 4.11 | 32.3 | .... |
| Recovery, % | 96 | 93 | .... |
| 1959 Commercial bleached: | | | |
| Total water-solubles | 4.01 | 61.1 | 1520 |
| Subfractions: | | | |
| Alcohol-soluble | 3.04 | 59.6 | 1960 |
| Alcohol-insoluble | 0.96 | 1.0 | 104 |
| Total | 4.00 | 60.6 | .... |
| Recovery, % | 100 | 99 | .... |
| Brevor bleached: | | | |
| Total water-solubles | 4.44 | 60.2 | 1360 |
| Subfractions: | | | |
| Alcohol-soluble | 3.48 | 58.2 | 1670 |
| Alcohol-insoluble | 0.97 | 0.8 | 83 |
| Total | 4.45 | 59.0 | .... |
| Recovery, % | 100 | 98 | .... |
| Elmar bleached: | | | |
| Total water-solubles | 4.06 | 70.7 | 1740 |
| Subfractions: | | | |
| Alcohol-soluble | 2.99 | 66.5 | 2220 |
| Alcohol-insoluble | 0.96 | 1.2 | 125 |
| Total | 3.95 | 67.7 | .... |
| Recovery, % | 97 | 96 | .... |

[a] Grams at 14% moisture obtained from 100.0 g. of flour at 14% moisture.
[b] Chloride in mg. per 100 g. of the fraction or flour indicated; all results are on a 14% moisture basis.

by the flour, approximately linear relations were found for any one fraction.

This evidence may also indicate that either the reaction occurred in two different phases or that there were two different reactions occurring, depending on the concentration of the chlorine.

*Chloride Distribution among Subfractions of the Water-Solubles.* Table VII shows that practically all the chloride of the water-solubles was in the low-molecular-weight material. Presumably this was free inorganic chloride ion. The water-soluble proteins and polysaccharides from unbleached flours had low levels of chloride and increased very little in chloride from bleaching. The chloride content of the original water-solubles as found in this manner agreed closely with those values reported in Table III.

*Chloride Distribution among Gluten Subfractions.* The crude

TABLE VIII
CHLORIDE DISTRIBUTION AMONG THE SUBFRACTIONS OF THE GLUTENS OF SOME CAKE FLOURS

| Flour, Treatment, and Fraction | Yield | Total Chloride | Chloride Content[a] |
|---|---|---|---|
| | g | mg | mg |
| 1959 Commercial unbleached: | | | |
| Original gluten | 1.00 | 0.2 | 17 |
| Subfractions: | | | |
| Butanol extract | 0.09 | 0.2 | 220 |
| Residue | 0.89 | 0.1 | 11 |
| Total | 0.98 | 0.3 | .... |
| Recovery, % | 98 | "150" | .... |
| 1959 Commercial bleached: | | | |
| Original gluten | 1.00 | 4.6 | 463 |
| Subfractions: | | | |
| Butanol extract | 0.07 | 2.9 | 4100 |
| Residue | 0.90 | 0.9 | 100 |
| Total | 0.97 | 3.8 | .... |
| Recovery, % | 97 | 83 | .... |
| Brevor bleached: | | | |
| Original gluten | 1.00 | 6.7 | 666 |
| Subfractions: | | | |
| Butanol extract | 0.10 | 3.2 | 3200 |
| Residue | 0.87 | 2.7 | 310 |
| Total | 0.97 | 5.9 | .... |
| Recovery, % | 97 | 88 | .... |
| Elmar bleached: | | | |
| Original gluten | 1.00 | 9.4 | 937 |
| Subfractions: | | | |
| Butanol extract | 0.05 | 4.3 | 8600 |
| Residue | 0.94 | 2.6 | 280 |
| Total | 0.99 | 6.9 | .... |
| Recovery, % | 99 | 72 | .... |

[a] Chloride in mg. per 100 g. of the fraction or flour indicated.

lipid portion (butanol extract) contained the major part of the gluten chloride as shown in Table VIII. The crude lipids from the bleached glutens reached 3 to 9% chloride contents (3,000 to 9,000 mg. chloride). However, the protein residues retained a substantial part of the gluten chloride.

## Discussion

Prime starch isolated from bleached flour had approximately the same chloride content as prime starch isolated from unbleached flour. Increased chloride contents were observed in the water-soluble and gluten fractions isolated from bleached flour. The large increase in water-soluble chloride may indicate that a reaction occurs with some flour component in which chlorine serves as an oxidizing agent and is reduced to the chloride ion. No preferential uptake of chlorine by gluten or water-solubles was evident.

The possibility exists that chlorine may be oxidizing the prime starch, thus creating new reactive sites within the starch polymer. Further work to investigate possible oxidation of starch is being undertaken.

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# THE BIURET TEST AS APPLIED TO THE ESTIMATION OF WHEAT PROTEIN[1]

ALVIN J. PINCKNEY[2]

## ABSTRACT

The biuret test developed by the author in 1949 for estimating protein content in wheat and flour has been modified to make it simpler and more accurate. Formerly, aliquots of cleared alkaline protein extracts were combined with measured amounts of the alkaline copper reagent. In the modified method the stabilized alkaline copper reagent is applied directly to the weighed sample for simultaneous extraction and reaction. Color intensities of the centrifuged test solutions, as determined in a spectrophotometer, are closely correlated with the protein contents of the samples as determined by the Kjeldahl method. Comparative tests indicate that sodium potassium tartrate is superior to glycerol as a stabilizer for copper in the biuret reagent.

During recent years a number of simple quantitative tests for protein in wheat and flour have been introduced. Among these, the biuret test as developed by the author (3) in 1949 has attracted favorable attention. In the original procedure, the protein extracted from the sample by shaking for 10 minutes with dilute alkali was treated with the alkaline copper reagent. In the procedure described in the present paper, the alkaline copper reagent serves also as the extractant, so that extraction and reaction proceed simultaneously. Thus, extraction is more nearly complete, and two volumetric measurements are eliminated. In older biuret methods, copper is held in solution by strong alkali for more or less complete reaction with the protein. But the copper tends to precipitate as the hydroxide before the reaction is complete. A more reliable result can be obtained if the copper is more completely stabilized by one of several possible agents. Ethylene glycol was proposed for this purpose by Mehl (2) in 1945. Sols (5) in 1947 employed glycerol. This agent was also used by the author in 1949 and in the present studies.

Jennings (1) has suggested sodium potassium tartrate as a copper stabilizer. This agent is used in the well-known Fehling test for sugar.

In this paper, there are presented the results of some biuret protein tests on samples of wheat and flour selected from the wheat classes hard red spring, hard red winter, soft red winter, white, and durum.

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These studies were designed to show the usefulness of the test in estimating protein when using glycerol or sodium potassium tartrate as the copper stabilizer in the biuret reagent. The protein content of the samples was determined by the usual Kjeldahl method.

## Method

*Apparatus:* (1) Balance, analytical.
(2) Shaker; motor-driven rack which inverts stoppered centrifuge tubes about 60 times per minute.
(3) Centrifuge; Clay-Adams "Senior"[a]; speed, 4,000 r.p.m.
(4) Mill, Labconco.
(5) Spectrophotometer; Coleman Jr., Model 6A, fitted for ½-in. square cuvets.

*Reagents:*
A. Glycerol reagent. To 937 ml. distilled water, add 10 ml. of 10*N* potassium hydroxide solution and 3.0 ml. of glycerol. Add slowly with vigorous stirring, 50 ml. of 4% $CuSO_4 \cdot 5H_2O$ solution.
B. Sodium potassium tartrate reagent. To 930 ml. of distilled water, add 10 ml. of 10*N* potassium hydroxide solution and 20 ml. of 25% sodium potassium tartrate solution. Add slowly with vigorous stirring, 40 ml. of 4% $CuSO_4 \cdot 5H_2O$ solution.

In preparing the reagents, copper hydroxide may be precipitated if the solution is not stirred vigorously during addition of the copper sulfate. The reagents should be discarded if they are not perfectly clear and free of sediment.

*Procedure:* Weigh into an 80-ml. centrifuge tube (or other suitable container) 0.6 g. of sample for use with reagent A or 0.5 g. for use with reagent B. Mix with 1 ml. of carbon tetrachloride, and then add 50 ml. of the indicated reagent. Stopper and shake vigorously for 10 minutes, let stand 1 hour, and then mix thoroughly. Transfer a portion to a 20-ml. centrifuge tube and centrifuge until perfectly clear. Determine the color intensity at 550 $m\mu$ (3) in the spectrophotometer with the indicated reagent as the blank.

In establishing a curve or chart to convert biuret to protein values, a regression equation should be derived (4) from the biuret and Kjeldahl protein values of 35 or more samples selected to represent the normal protein range.

## Experimental

Each of the reagents, A and B, was used in testing weighed portions of a sample of flour containing 17.5% protein. The weights of these portions were varied to represent the protein range 2 to 20%. In addition, a third reagent was used. This reagent, designated as "modified A," was similar to reagent A but contained only 40 ml. of copper solution per liter, as in reagent B. From the paired values, absorbance and percent protein, the regressions were calculated. These are shown in Table I.

The starch in wheat and flour samples adsorbs some of the copper. This reduces the color of the reagent and the observed absorbance

[a]Mention of specific trade names or instruments is made for the purpose of identification and does not imply any endorsement by the United States Government.

of the test solution. In order to determine the magnitude and net effect of this factor, the reagents were applied to testing purified wheat starch. A portion of each reagent was diluted with 10*N* potassium hydroxide solution, three parts to one part of reagent. Portions of the starch weighing 0.4 g. were then tested with each of the reagents, diluted and undiluted. In determining the color intensities in the spectrophotometer, a water blank was used. The results are shown in Table II.

In the previous paper (3), it was found that about 85% of the total protein (Kjeldahl) in wheat and about 95% of the total protein in flour were extracted in the test. In applying the modified method, the protein concentration in the test solutions of eight samples of wheat and four samples of flour were determined by the Kjeldahl

TABLE I

REAGENT COLOR INTENSITY AND RESPONSE

| Reagent | Water Blank: Reagent Color Intensity Absorbance[a] | Reagent Blank | | | |
|---|---|---|---|---|---|
| | | Regression Equation: Absorbance × % Protein | Absorbance at 14% Protein Level | r | Sy.x |
| A | 0.170 | $y = 34.6x - 0.5$ | 0.419 | 0.9994 | ± 0.21 |
| Modified A | 0.150 | $y = 40.5x - 0.2$ | 0.350 | 0.9996 | ± 0.16 |
| B | 0.050 | $y = 38.3x - 0.4$ | 0.376 | 0.9995 | ± 0.19 |

[a] Protein calculated on the assumption 0.6-g. sample for reagent A or 0.5-g. sample for modified A and for reagent B corresponds to 17.5, the percent protein in sample. Thus, 0.1-g. sample = 1/6 × 17.5 = 2.9%, for A, or 1/5 × 17.5 = 3.5% for modified A and for B.

TABLE II

ADSORPTION OF COPPER BY STARCH

| | Glycerol Reagent | | NaK Tartrate Reagent | |
|---|---|---|---|---|
| | Undiluted | Diluted | Undiluted | Diluted |
| Reagent absorbance | 0.170 | 0.051 | 0.053 | 0.023 |
| Starch test absorbance | 0.141 | 0.024 | 0.048 | 0.021 |
| Difference | 0.029 | 0.027 | 0.005 | 0.002 |
| Equivalent protein, % | 1.5 | 1.4 | 0.5 | 0.4 |

TABLE III

PERCENTAGE OF THE TOTAL PROTEIN (KJELDAHL) EXTRACTED BY THE REAGENTS

| | Wheat | | | | Flour | |
|---|---|---|---|---|---|---|
| | Hard Red Winter | | Durum | | Hard Red Spring | |
| | Reagent A | Reagent B | Reagent A | Reagent B | Reagent A | Reagent B |
| | 88.4 | 89.4 | 86.0 | 89.2 | 99.6 | 98.9 |
| | 89.5 | 91.0 | 87.6 | 90.8 | 100.0 | 100.0 |
| | 89.7 | 92.2 | 88.8 | 91.0 | 100.0 | 100.0 |
| | 89.2 | 92.9 | 90.4 | 92.3 | 100.0 | 100.0 |
| Average | 89.2 | 91.4 | 88.2 | 90.8 | 99.9 | 99.7 |

method. These values are shown in Table III as percentages of the total protein of the samples.

A statistical summary of the results obtained in applying the modified biuret test to the samples selected from the five wheat classes is presented in Table IV. For comparison, certain values from the previous paper are also included.

The errors in the predicted protein values of the 24 hard red winter wheat samples selected for the present study and of the 100 hard red winter wheat samples in the previous work (3) have been calculated and classified as shown in Table V.

TABLE IV

STATISTICAL SUMMARY OF THE RESULTS OBTAINED IN TESTING SAMPLES OF THE FIVE MAIN CLASSES OF WHEAT AND OF FLOUR OF HARD RED SPRING WHEAT

| Reagent and Material | N | Regression | r | Sy.x | σ (Protein) |
|---|---|---|---|---|---|
| Glycerol (a) | | | | | |
| Wheat: HRS | 32 | $y = 31.4x + 0.4$ | 0.99 | ± 0.28 | 2.4 |
| HRW | 24 | $y = 32.8x + 0.7$ | 0.97 | ± 0.21 | 0.8 |
| SRW | 24 | $y = 30.9x + 0.9$ | 0.99 | ± 0.12 | 1.1 |
| White | 19 | $y = 28.1x + 2.0$ | 0.94 | ± 0.27 | 0.8 |
| Durum | 30 | $y = 38.9x - 1.6$ | 0.98 | ± 0.28 | 1.4 |
| Durum[a] | 30 | $y = 37.5x - 1.8$ | 0.99 | ± 0.21 | 1.4 |
| Flour: HRS | 24 | $y = 27.7x + 1.6$ | 0.99 | ± 0.20 | 1.7 |
| NaK Tartrate (b) | | | | | |
| Wheat: HRS | 32 | $y = 39.0x - 0.7$ | 0.99 | ± 0.21 | 2.4 |
| HRW | 24 | $y = 40.9x - 0.9$ | 0.98 | ± 0.18 | 0.8 |
| SRW | 24 | $y = 38.8x - 0.2$ | 0.99 | ± 0.15 | 1.1 |
| White | 19 | $y = 36.3x + 0.3$ | 0.95 | ± 0.26 | 0.8 |
| Durum | 30 | $y = 47.2x - 2.5$ | 0.99 | ± 0.18 | 1.4 |
| Durum[a] | 30 | $y = 41.8x - 1.1$ | 0.99 | ± 0.16 | 1.4 |
| Flour: HRS | 24 | $y = 39.5x - 0.9$ | 0.99 | ± 0.23 | 2.0 |
| Glycerol, 1949[b] (Wheat HRW) | 100 | ............. | 0.96 | ± 0.38 | 2.0 |

[a] Shaken 15 minutes.
[b] From previous paper, 1949 (ref. 3).

TABLE V

PERCENTAGE OF SAMPLES IN EACH OF FIVE SELECTED RANGES OF ERROR IN PREDICTED PROTEIN CONTENT (HARD RED WINTER WHEAT)

| Protein Error Range | Percentage of Samples in Each Range | | |
|---|---|---|---|
| | Reagent A | Reagent B | 1949 Method |
| 0.0 – 0.19% | 70.8 | 79.2 | 41.0 |
| 0.2 – 0.39% | 20.8 | 16.6 | 35.0 |
| 0.4 – 0.59% | 8.4 | 4.2 | 17.0 |
| 0.6 – 0.79% | 0.0 | 0.0 | 6.0 |
| 0.8 – 0.89% | 0.0 | 0.0 | 1.0 |
| Sum | 100.0 | 100.0 | 100.0 |

## Discussion

The data in Table I indicate that the amounts of copper in the reagents as applied are adequate for the protein range 0 to 20%. Similar experiments have shown that less than two-thirds of the copper is taken up by the protein at the 20% level.

As observed by Jennings (1), the color intensity of the glycerol reagent is greater than that of the sodium potassium tartrate reagent (Tables I and II). Consequently, the adsorption of copper by starch causes a greater decrease in the color intensity of the glycerol reagent than in that of the tartrate reagent (Table II). The concentration of the copper in the diluted reagents (Table II) is less than that of the residual copper in the test as ordinarily applied to a sample containing 24% protein. Thus, for each of the reagents, A and B, the starch effect is nearly constant throughout the protein range.

The differences in color intensity change from this cause are probably reflected by the differences in *a* values ($y = bx + a$) of the regressions involving the two reagents (see Table IV).

In Table I, the difference in average absorbances for the two series seems to indicate a greater color response for the sodium potassium tartrate reagent, as stated by Jennings (1). The test solution contains two color components: the protein-copper complex and the uncombined copper. The color intensity of the latter is diminished from its original value by the transfer of the copper to the protein. The instrument zero, adjusted with a reagent as the blank, is in error by an amount proportional to the amount of protein in the sample tested and also proportional to the color intensity of the reagent used. The absorbance of each test represents less than the total of the color intensity of the biuret complex by the amount of this error. Therefore, the color responses of the two reagents may be nearly identical.

From Table III, it is evident that the protein of durum is slightly more resistant to extraction than is the protein of wheat other than durum. Apparently, the tartrate reagent is slightly the more effective in extracting the protein from wheat.

In Table IV the regressions of the classes other than durum are similar within each reagent series. The regressions for the class durum are quite different. The longer shaking time of the second durum series produced regressions which are more nearly in line with those of the other classes. The change in the regression slope for the glycerol series is comparatively slight, but it is accompanied by corresponding changes in r, Sy.x, and average absorbances. The latter value increased from 0.450 to 0.472 for the glycerol series and from

0.391 to 0.408 for the tartrate series.

The modified method is more accurate than the original method (3), as shown by the more favorable r and Sy.x values and error distribution (Table V). By the same standards, the sodium potassium tartrate reagent is preferable to the glycerol reagent.

The modified biuret method gives a means of estimating wheat protein in a fairly satisfactory manner. It is simpler than the original method, and the results are more nearly in line with the Kjeldahl values. The method is empirical, and a strict time schedule must be followed to get good results.

The advantages of the tartrate reagent over the glycerol reagent largely result from the lesser reagent color intensity.

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# IDENTIFICATION OF CARBONYL COMPOUNDS PRODUCED IN PRE-FERMENTS[1]

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## ABSTRACT

Extraction of a pre-ferment with diethyl ether or of the condensed gases with carbon tetrachloride was ineffective for obtaining sufficient minor flavor constituents for analysis by gas chromatography using thermistors as detectors. Passing the natural gases from a pre-ferment through 2,4-dinitrophenylhydrazine reagent was a convenient way of isolating carbonyl compounds in reasonable quantity. Nitrogen also was effective for sweeping the carbonyl compounds from a pre-ferment.

Formaldehyde, acetaldehyde, acetone, isobutyraldehyde, n-butyraldehyde, methylethylketone, isovaleraldehyde, and n-valeraldehyde were detected by gas chromatography of the precipitated carbonyl derivatives by heating the mixed hydrazones in the presence of alpha-ketoglutaric acid. All of these but n-valeraldehyde were isolated from the precipitated mixture by passing it through a Celite column. In addition, 2-methyl-1-butanal was isolated by column chromatography. Individual components were identified by comparing them with authentic compounds using paper chromatography in two different solvents and by ultraviolet and infrared spectral analyses.


There is much interest in pre-ferments and their use in continuous processes for breadmaking. Some, however, consider the flavor of bread made by the continuous processes inferior to the flavor produced by the customary sponge process. Efforts are needed, therefore, to improve flavor characteristics of pre-ferments used in continuous processes. Since much of bread flavor originates during fermentation and since alterations in processing provide opportunities to modify or intensify flavor, knowledge concerning flavors produced in pre-ferments is essential to advance continuous breadmaking methods and to improve the organoleptic qualities of bread.

The small concentration of flavor compounds in pre-ferments makes analyses difficult. Even with the most sensitive methods, such as gas chromatography, it is necessary to concentrate the compounds by extraction, distillation, formation of derivatives, or by column or paper chromatography. Extreme care must be exercised while pre-ferments or pre-ferment extracts are being concentrated because of the volatile nature of flavor components and because of possible

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interactions of certain components.

Johnson, Miller, and Curnutte (5) and Smith and Coffman (13) have investigated chemical compounds produced in pre-ferments. Using paper and column chromatography, Johnson, Miller, and Curnutte (5) analyzed for organic acids and esters and obtained some quantitative data. Using gas chromatography, Smith and Coffman (13) separated and identified several of the neutral components of pre-ferment. Neish and Blackwood (6) determined the organic acids and certain other compounds produced during the dissimilation of glucose by yeast. One of the carbonyl compounds measured quantitatively was acetoin (6). Visser't Hooft and de Leeuw (14) demonstrated that acetoin is slowly oxidized to diacetyl, but because of its high volatility, little diacetyl was found in the finished bread.

Ng, Reed and Pence (7) and Wiseblatt and Kohn (15) examined baked bread for compounds that may be responsible for bread flavor. It is likely that several of the flavor compounds in bread arise from the baking, whereas others originate during fermentation (9). This paper describes the isolation and qualitative identification of several volatile compounds, mainly carbonyls, which are present in pre-ferments.

## Materials and Methods

The pre-ferment used throughout this work was the salt-buffered type employed by Robinson *et al.* (11). The analyses of pre-ferments for constituents involved extraction with ether after 4 hours of fermentation, collection of gases evolved during fermentation, and formation of derivatives from extracted or collected carbonyl compounds. Further isolation and identification of flavor components were based on paper, column, and gas chromatography supplemented with ultraviolet and infrared spectroscopy.

*Gas Chromatography.* The gas chromatography unit used was a Perkin-Elmer Vapor Fractometer Model 154-C[2] equipped with thermistors. The columns were ¼-in. × 6-ft. tubes packed with the materials noted in Tables I and II. The experimental conditions for gas chromatography are noted in Tables I and II.

*Extraction of Pre-ferments with Diethyl Ether.* A 200-ml. portion of freshly centrifuged pre-ferment was extracted in a separatory funnel with 40 ml. of diethyl ether. This ether layer was removed and added to a fresh 200-ml. aliquot of pre-ferment along with 20 ml. of additional ether. Extraction was completed as for the first portion

[2]The mention in this publication of a trade product, equipment, or a commercial company does not imply its endorsement by the U.S. Department of Agriculture over similar products, companies, etc., not named.

and the process repeated until 1,200 ml. of pre-ferment were extracted. The ether layer from the last 200-ml. aliquot was centrifuged, and the ether was decanted into a glass-stoppered vial and dried over anhydrous sodium sulfate before being analyzed in the gas chromatograph, using 30-$\mu$l. quantities.

*Extraction of Condensate from Evolved Gases with Carbon Tetrachloride.* Sixteen liters of pre-ferment (11) were allowed to ferment in a 5-gal. polyethylene bottle connected in series by tubing to two 1-liter wash bottles in Dewar flasks. The first bottle was surrounded by an ice-salt mixture and the second by a dry ice-acetone mixture. The liquid (approximately 12 ml.) in the first trap was added to the dry-ice trap and the resulting solution extracted in a separatory funnel with 5 ml. of carbon tetrachloride. The separated solvent was stored in a glass-stoppered vial over anhydrous sodium sulfate and placed in a deep freeze until analyzed by gas chromatography using 30 $\mu$l. quantities. Carbon tetrachloride was chosen as a solvent because of its high density and stability and because all impurities could be removed by repeated distillations.

*Confirming the Presence of Carbonyl Compounds in Extracts.* To demonstrate the presence of carbonyl compounds in the extracts, a 1-ml. portion was shaken intermittently for 30 minutes with 1 ml. of water containing 100 mg. of sodium bisulfite (1). This treatment resulted in the formation of a complex between the bisulfite and the carbonyl compounds and the elimination of the peaks due to carbonyl compounds in the gas chromatogram. Most of the water was removed by freezing in a deep freeze for 15 minutes and the remainder by drying over anhydrous sodium sulfate. Thirty-microliter quantities were analyzed by gas chromatography.

*Formation of Hydrazones from Carbonyl Compounds in Evolved Gases.* Derivatives of carbonyl compounds formed during the first 6 hours of fermentation of a 16-l. pre-ferment were prepared by passing the gases first through a wash bottle surrounded by an ice-salt bath and then through 250 ml. of a filtered 2,4-dinitrophenylhydrazine solution prepared by stirring 3 g. of the solid reagent in 500 ml. of 2*N* hydrochloric acid. The solution containing the precipitated 2,4-dinitrophenylhydrazones (hereafter abbreviated 2,4-DNPH's) was cooled at 5°C. for 3 hours and filtered. When the filtrate was extracted twice with two-fifths the volume of ethyl acetate, a second batch of hydrazones was obtained. When the filtrate was stored at 5°C. several days, a third group of light-yellow crystals formed.

It was also possible to remove the carbonyl compounds which remained in the pre-ferment after active fermentation. This was

achieved by adding mercuric chloride to prevent further fermentation and sweeping the pre-ferment with nitrogen gas which was then passed through the 2,4-dinitrophenylhydrazine reagent. The carbonyl compounds isolated were the same as those isolated during active fermentation. Quantitative analyses of the flavor compounds isolated in this manner should be indicative of the quantities present in pre-ferments used commercially.

*Separation and Identification of Carbonyl Hydrazones.* The carbonyl hydrazones were separated by two different techniques, both of which employed the recrystallized solids separated from the 2,4-dinitrophenylhydrazine reagent. One method involved separating individual hydrazones by column partition chromatography (4), using Hyflo Super Cel in place of Celite Analytical Filter-aid because satisfactory separation was obtained in one-fifth the time. The second method involved gas chromatography of the volatile carbonyl compounds which were liberated from these hydrazones by heating in the presence of alpha-ketoglutaric acid (10).

Identification of the carbonyl compounds separated by column chromatography (4) was also accomplished by descending chromatography using Whatman No. 1 paper. The solvents used were 2-phenoxyethanol-heptane and N,N-dimethylformamide-decalin (7).

The ultraviolet spectrum analyses of the separated 2,4-DNPH's were made on a Beckman (Model DU) spectrophotometer by two methods. Absorbance was measured by using the chromatographed paper spot of the respective carbonyl compound (8) and by using chloroform solution of the eluted purified carbonyl compounds. The latter method was more reproducible and reliable.

The infrared spectrum analyses of the separated and purified 2,4-DNPH's and known carbonyl compounds were obtained by the potassium bromide technique (13-mm. pellets) and a Perkin-Elmer Model 137 Infracord spectrophotometer. In cases where only micro quantities of 2,4-DNPH's were available a 1.5-mm. pellet was employed.

*Preparation of Known Carbonyl Derivatives.* The 2,4-DNPH's of known carbonyl compounds were prepared and purified by the techniques of Shriner, Fuson, and Curtin (12).

## Results and Discussion

*Flavor Components in Ethyl Ether Extracts of Pre-ferments.* The ether extracts of pre-ferments were analyzed by gas chromatography with the results shown in Table I. Ethyl alcohol was the main component, but lesser amounts of ethyl acetate, isobutyl alcohol, and

TABLE I

FLAVOR COMPOUNDS DETECTED BY GAS CHROMATOGRAPHY IN AN ETHYL ETHER EXTRACT OF PRE-FERMENTS AND IN A CARBON TETRACHLORIDE EXTRACT OF EVOLVED GAS CONDENSATE FROM PRE-FERMENTS

| Identified Compounds in Pre-ferments | Retention Time | | | |
|---|---|---|---|---|
| | Column A[a] | | Column X[b] | |
| | Compound in Extract | Authentic Compound | Compound in Extract | Authentic Compound |
| | *minutes* | *minutes* | *minutes* | *minutes* |
| Compounds extracted from pre-ferment with ethyl ether | | | | |
| Ethyl alcohol | 3.0 | 3.0 | ...[c] | ...[c] |
| Ethyl acetate | 5.6 | 5.6 | 12.6 | 12.6 |
| Isobutyl alcohol | 9.3 | 9.3 | 21.7 | 21.3 |
| Isoamyl alcohol | 20.5 | 21.2 | 47.8 | 48.0 |
| Compounds present in carbon tetrachloride extract of dry ice-acetone condensed gases | | | | |
| Acetaldehyde | 2.0 | 1.9 | 4.5 | 4.4 |
| Ethyl alcohol | 3.0 | 3.0 | 7.0 | 6.8 |
| Ethyl acetate | 5.7 | 5.6 | 12.4 | 12.6 |
| Isoamyl alcohol | 21.2 | 21.2 | 48.2 | 46.7 |
| Solvents | | | | |
| Diethyl ether | | 1.9 | | 4.2 |
| Carbon tetrachloride | | 7.5 | | 16.4 |

[a] Celite 545 (60–80 mesh) coated with 20% by weight of diisodecyl phthalate; column at 98°C.; helium flow 32 ml. per minute.
[b] Celite 545 (80–100 mesh) coated with 30% by weight of di(2-ethyl-hexyl)phthalate; column at 98°C.; helium flow 11.3 ml. per minute.
[c] Same retention time as ether.

isoamyl alcohol were present. Columns A and X both detected the same compounds, although ethyl alcohol and ether emerged together in column X. Using column F, isoamyl alcohol was not detected, probably because of the extremely long retention time (95 minutes) of this compound. Micro amounts of compounds retained a long time generally are released slowly and exhibit only a very small millivolt response over several minutes.

These data confirm the presence of four of the twenty-seven compounds isolated from a pre-ferment by Smith and Coffman (13), using an elaborate distillation and concentration procedure. Undoubtedly, if a more sensitive detector, such as a hydrogen flame device, were used many more components would have been detected. Because of the inadequacy of simple ether extraction of the pre-ferment and the inability of the thermistors to detect more than a few compounds, other procedures were employed to study compounds which were extremely volatile or present in micro amounts.

*Flavor Components in Carbon Tetrachloride Extracts of Condensed Gases from Pre-ferments.* The compounds detected in the condensed gases (Table I) were somewhat different from those de-

tected in the ether extracts. Acetaldehyde was found because this highly volatile substance was retained at the cold temperature of the acetone-dry ice trap. Ethyl alcohol, ethyl acetate, and isoamyl alcohol were detectable but isobutyl alcohol was not. Acetaldehyde and isoamyl alcohol also were detected using column F (described in Table II), but ethyl alcohol and ethyl acetate emerged with carbon tetrachloride in this column. All these compounds were also detected by Smith and Coffman (13).

*Carbonyl Compounds in Gases Evolved from Pre-ferments.* The mixed 2,4-DNPH's obtained from the gases of the pre-ferment were analyzed by two methods. The simplest technique used was that of Ralls (10) employing the gas chromatograph to detect carbonyl compounds released from their 2,4-DNPH's by heating in the presence of alpha-ketoglutaric acid. The eight compounds (Table II) detected by

TABLE II

CARBONYL COMPOUNDS PRESENT IN GASES EVOLVED FROM PRE-FERMENTS, MADE INTO 2,4-DNPH DERIVATIVES, AND DETECTED BY GAS CHROMATOGRAPHY[a]

| Identified Compounds in Pre-ferments | Retention Time | | | | | |
|---|---|---|---|---|---|---|
| | Column A[b] | | Column X[c] | | Column F[d] | |
| | Compound in Gases | Authentic Compound | Compound in Gases | Authentic Compound | Compound in Gases | Authentic Compound |
| | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* |
| Formaldehyde | 1.7 | 1.6 | 3.2 | 3.2 | 1.0 | 1.1 |
| Acetaldehyde | 1.9 | 1.9 | 4.4 | 4.4 | 3.0 | 3.1 |
| Acetone | 3.5 | 3.6 | 7.7 | 7.7 | 6.9 | 6.8 |
| Isobutyraldehyde | 4.7 | 4.8 | 10.3 | 10.2 | 8.3 | 8.5 |
| n-Butyraldehyde | 5.7 | 5.9 | 13.4 | 13.4 | 12.0 | 12.1 |
| Methylethylketone | 6.5 | 6.5 | 14.1 | 14.1 | 13.5 | 13.7 |
| Isovaleraldehyde | 9.5 | 9.4 | 19.9 | 19.9 | 18.6 | 18.5 |
| n-Valeraldehyde | 11.6 | 11.9 | 26.8 | 26.6 | 26.9 | 26.5 |

[a] See reference 10.
[b] Celite 545 (60–80 mesh) coated with 20% by weight of diisodecyl phthalate; column at 98°C.; helium flow 32 ml. per minute.
[c] Celite 545 (80–100 mesh) coated with 30% by weight of di(2-ethyl-hexyl)phthalate; column at 98°C.; helium flow 11.3 ml. per minute.
[d] Firebrick (30–60 mesh) coated with 25% by weight of tetraethyleneglycol dimethyl ether; column at 74°C.; helium flow 80 ml. per minute.

this technique contrast with only three volatile carbonyl compounds (acetaldehyde, acetone, and diacetyl) found in pre-ferments by Smith and Coffman (13). Acetoin, found by Smith and Coffman (13) in ether extracts of pre-ferments, was not found using the present technique because of its limited volatility (b.p. 188°C.).

The analysis of the 2,4-DNPH mixture by Ralls' technique (10) revealed that the amount of acetaldehyde 2,4-DNPH in the mixture was many times the concentration of the next most abundant com-

ponent. The small quantities of most of the 2,4-DNPH's made it impossible to identify the free carbonyls emerging from the column using infrared spectroscopy.

Techniques other than gas chromatography were necessary to separate sufficient quantities of most of the carbonyl compounds so that they might be identified by the use of ultraviolet and infrared spectroscopy. The technique which proved most useful for analyzing the mixed 2,4-DNPH's was a combination of their partial separation on a Celite column by the technique of Day, Bassette, and Keeney (3), followed by further purification by paper chromatography using N,N-dimethylformamide-decalin solvent. The ultraviolet spectra of the separated components were initially obtained directly on the air-dried paper chromatographs by the method of Nonaka, Pippen, and Bailey (8) and as used by Ng, Reed, and Pence (7). Results obtained by this method were highly erratic. Data obtained with the solvent extracts of the 2,4-DNPH spots were much more reliable. The purified carbonyl compounds were also identified by infrared spectra.

TABLE III

$R_H$ AND $R_F$ VALUES AND ULTRAVIOLET ABSORPTION MAXIMA FOR 2,4-DINITROPHENYLHYDRAZONES OF CARBONYLS ISOLATED FROM PRE-FERMENTS AND FOR COMPARABLE AUTHENTIC CARBONYL DERIVATIVES

| Carbonyl Compound | $R_H$ or $R_F$ Values of 2,4-DNPH[a] | | Absorption Maximum of 2,4-DNPH | |
|---|---|---|---|---|
| | Isolated | Authentic | Isolated | Authentic |
| | | | mμ | mμ |
| | 2-Phenoxyethanol:heptane solvent | | | |
| Formaldehyde | 0.17 | 0.16 | 363 | 364 |
| Acetaldehyde | 0.32 | 0.32 | 369 | 368 |
| Acetone | 0.32 | 0.31 | 375 | 374 |
| Methylethylketone | 0.49 | 0.49 | 374 | 374 |
| 2-Methyl-1-butanal | 0.60 | 0.60 | 356-7 | 356-7 |
| n-Butyraldehyde | 0.37 | 0.37 | 368 | 368 |
| iso-Butyraldehyde | 0.39 | 0.39 | 367 | 368 |
| iso-Valeraldehyde | 0.45 | 0.45 | 368 | 369 |
| | N,N-dimethylformamide:decalin solvent | | | |
| Formaldehyde | 0.12 | 0.12 | 362 | 362 |
| Acetaldehyde | 0.15 | 0.15 | 362 | 364 |
| Acetone | 0.25 | 0.26 | 368 | 368 |
| Methylethylketone | 0.38 | 0.38 | 377 | 376 |
| 2-Methyl-1-butanal | 0.68 | 0.69 | 368 | 368 |
| n-Butyraldehyde | 0.38 | 0.39 | 370 | 370 |
| iso-Butyraldehyde | 0.36 | 0.36 | 368 | 368 |
| iso-Valeraldehyde | 0.39 | 0.37 | 364 | 364 |

[a] $R_H$ values (distance of travel compared with that for 3-heptanone) are reported for the first solvent. $R_F$ values are reported for the second solvent.

Table III lists the $R_F$ and $R_H$ values in two different solvents and the maximum ultraviolet absorbances for the 2,4-DNPH's of carbonyl compounds isolated from the volatile gases of the pre-ferment. Similar data for corresponding known 2,4-DNPH's are also recorded. All the 2,4-DNPH's except formaldehyde and methylethylketone were isolated from the mixture separated by the first filtration of the 2,4-dinitrophenylhydrazine reagent. Formaldehyde and methylethylketone 2,4-DNPH's were isolated from the reagent by extraction with ethyl acetate. From all the mixed 2,4-DNPH crystals, at least seven carbonyl compounds were isolated but unidentified, in addition to the nine positively identified. Some of the unidentified compounds appeared to be unstable. The melting points of isolated acetaldehyde and methylethylketone 2,4-DNPH's were 162° and 119°C., respectively. These agree well with the values of 160° and 120°C. for similar derivatives of authentic acetaldehyde and methylethylketone. Other 2,4-DNPH's of carbonyl compounds found in the pre-ferment were not crystallized in sufficient quantities to permit the determination of melting points.

The 2,4-DNPH's isolated by column and paper chromatography agreed with those identified by gas chromatography (Table II), except for 2-methyl-1-butanal and n-valeraldehyde. The 2-methyl-1-butanal was not identified in the 2,4-DNPH mixture by gas chromatography. N-valeraldehyde could not be distinguished from isovaleraldehyde by paper chromatography, even though two different solvents were employed. The maximum ultraviolet absorbances of the isolated 2,4-DNPH's agreed well with those for authentic samples. However, this technique failed to distinguish among the aldehydes and between the ketones.

The identification of all but two of the compounds in Table III was confirmed by infrared spectra. Insufficient 2,4-DNPH's of formaldehyde and 2-methyl-1-butanal were obtained for identification by this technique. The identity of formaldehyde 2,4-DNPH, however, was confirmed using chromotropic acid (2).

Wiseblatt and Kohn (15) isolated acetaldehyde, acetone, 2-ethyl hexanal, 2-hexanone, 3-heptanone, crotonaldehyde, diacetyl, methyl glyoxal, and furfuraldehyde from fresh bread. The only carbonyls of this group found in pre-ferments during the present investigation were acetaldehyde and acetone. Diacetyl and pyruvaldehyde (methyl glyoxal) are probably present among the unidentified carbonyls produced during fermentation. The other carbonyls found by Wiseblatt and Kohn (15) may result from the browning reactions during baking and processing.

Ng, Reed, and Pence (7) found formaldehyde, acetaldehyde, acetone, methylethylketone, ethyl pyruvate, 2-methyl-1-butanal, hexanal, and furfural in the ethanol extract of bread. They also found isobutyraldehyde and n-valeraldehyde in vapors of baking bread, but not in the ethanol extract of the bread crumb. In this work, most of these compounds were found in pre-ferments. Exceptions were furfural, which probably results from the browning reaction during baking, hexanal, and ethyl pyruvate which may be among the unidentified carbonyl compounds.

### Acknowledgments

The authors gratefully acknowledge the assistance of John W. Sullivan in preparing infrared absorption curves used in the identification of the carbonyl compounds, and the kindness of J. W. Pence, Western Utilization Research and Development Division, for supplying a sample of 2-methyl-1-butanal 2,4-DNPH. The pioneering work of James F. Lawrence with gas chromatography of pre-ferment extracts and the financial assistance of Corn Products Sales Company, New York, N. Y., are also gratefully acknowledged.

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# CHLOROETHANOL AS A CEREAL PROTEIN DISPERSANT[1]

N. W. Tschoegl[2]

## ABSTRACT

Wheat gluten may be dispersed virtually completely in a 0.01*N* solution of hydrochloric acid in 70% aqueous 2-chloroethanol, from which it may be recovered by precipitation with diethyl ether with its properties apparently unaltered. A 0.1*N* solution of hydrogen chloride in anhydrous chloroethanol also disperses wheat gluten completely, but there is some loss of amide nitrogen and some of the free carboxyl groups become esterified. After precipitation with diethyl ether the cohesive properties of the gluten are lost. Anhydrous acid chloroethanol may be used also in the direct extraction of the proteins from wheat and rye flour, but is less effective in the extraction of barley and oat proteins. The extraction of the protein from the germ flour of the carob bean *(Ceratonia siliqua)* is still less complete. Acid-free anhydrous chloroethanol disperses about 60% of wheat gluten. Aqueous acid chloroethanol is about as efficient as anhydrous acid chloroethanol for wheat but extracts more oat flour protein.

The intrinsic (bulk) viscosity of gluten dispersed in aqueous acid chloroethanol is much higher than that of gluten dispersed in anhydrous acid chloroethanol, but the reverse is true in regard to the limiting surface viscosities of gluten spread from these dispersions at an oil/water interface.

The investigation reported in this paper arose from a study of spread monolayers of wheat gluten and other cereal proteins (15,16), in particular the choice of a suitable dispersion medium for spreading purposes. Dispersions in dilute acids and in sodium salicylate were the obvious first choice. From the former, however, gluten could not be spread without the aid of a spreading agent. Furthermore, dispersions of gluten in dilute acids are known (14) to be very polydisperse. Gluten was found to spread readily from dispersions in 10% w/v sodium salicylate, the salicylate itself apparently acting as the spreading agent, but the films gave rather low surface viscosities.

Attention was therefore turned to the chance observation by Hess (9) that native wheat flour protein could be dispersed completely in 2-chloroethanol and recovered by precipitation with diethyl ether with its properties apparently unaltered. When this observation was checked, virtually complete dispersion of vacuum-dried gluten was obtained in a clear but not specially purified laboratory sample of chloroethanol. The gluten particles first swelled, then disintegrated, and were completely dispersed on shaking after about 3 days. Centrifugation removed some starch and also some

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protein, but most of the latter could be redispersed by shaking up the residue with a few ml. of the dispersant. The supernatant was much less turbid than a dispersion of the same concentration in sodium salicylate and gave no iodine reaction. Gluten films could be spread readily from a suitable dilution and showed much higher surface viscosities than films spread from dispersion in sodium salicylate. However, when the experiment was repeated with a carefully purified sample of chloroethanol, only about 60% of the gluten would disperse.

As chloroethanol appeared to be an attractive dispersion medium for surface chemical studies in particular and physicochemical investigations in general, some of the factors influencing its gluten-dispersing power were investigated. These investigations were then extended to the direct extraction of the proteins from wheat flour and from the flours of rye, barley, and oat, and the germ flour of the carob bean *(Ceratonia siliqua)*. Carob germ flour was included since it (and perhaps that of some related species (3)) is apparently the only known nonwheat-plant protein to possess gluten-forming ability (12,13).

## Materials and Methods

Reagents were of A.R. quality unless otherwise stated. Water was first deionized and then distilled from an all-glass still containing a trace of sulfuric acid.

*Flours.* Wheat flours were milled on a Buhler experimental mill from sound whole grain of known variety. Rye, barley, and oat flours were milled on an Allis-Chalmers mill from whole rye, pearled barley, and dehulled oats.

Carob bean seeds were softened by boiling in water for 30 minutes and soaking in a fresh portion of water overnight. They were then opened by hand, and the germ was picked out and dried over anhydrous calcium chloride. This procedure was thought to be preferable to that of Rice and Ramstad (12) in which the seed coats are carbonized in concentrated sulfuric acid. The germ was ground in a laboratory hammer mill and bolted through 56-mesh nylon gauze. The samples were analyzed by the official methods of the AOAC (2) for protein, moisture, ash, and fat. Doughs made from the wheat flours were tested on the Chopin Alveograph. The analytical and physical test data are shown in Table I.

*Preparation of Gluten.* Gluten was washed from the medium wheat flour following the standard procedure detailed in *Cereal Laboratory Methods* (1), except that Dill and Alsberg's (6) phosphate

TABLE I

ANALYTICAL DATA OF FLOURS

| Analytical Measurement | Flour | | | | | | |
|---|---|---|---|---|---|---|---|
| | Strong Wheat | Medium Wheat | Weak Wheat | Rye | Barley | Oat | Carob Germ |
| | % | % | % | % | % | % | % |
| Protein (dry basis) | 11.71 | 12.50 | 9.92 | 6.52 | 6.15 | 8.20 | 62.2 |
| Moisture | 15.0 | 14.4 | 12.8 | 13.4 | 12.2 | 9.2 | 9.3 |
| Ash (dry basis) | 0.55 | 0.48 | 0.53 | 0.38 | 0.59 | 0.74 | ... |
| Fat (dry basis) | 1.91 | 1.80 | 2.06 | 1.56 | 1.70 | 8.68 | 9.20 |
| Alveogram "strength" | 84 | 48 | 13 | ... | ... | ... | ... |

buffer was substituted for tapwater. The gluten was dried *in vacuo,* powdered, bolted through 56-mesh nylon gauze, and stored over phosphorus pentoxide in a vacuum desiccator. The protein content of the dried gluten was about 80%.

*Dispersion Media.* 2-Chloroethanol was purified from laboratory-grade material. After a preliminary distillation under reduced pressure, the sample was carefully dried and freed from acid over desiccated potassium carbonate and twice redistilled under a vacuum of about 18–22 mm. Hg. The purified chloroethanol had an acidity of less than 0.005% w/v expressed as hydrochloric acid and a water content of less than 0.5% w/v. Solutions of hydrogen chloride in purified chloroethanol were prepared by passing in dry hydrogen chloride gas (19) until a concentration of 0.365% (0.1*N*) was attained. Solutions of lower acidity were obtained by dilution of the 0.1*N* sample with purified chloroethanol. Aqueous acid chloroethanol solutions were prepared by diluting anhydrous acid chloroethanol of the required acidity with hydrochloric acid of the same strength.

Solutions of other acids in purified chloroethanol were obtained by adding the required amounts of concentrated sulfuric, anhydrous (90%) formic, glacial acetic, or trichloroacetic acid.

Solutions of acids in alcohols were similarly prepared from redistilled n-butanol, isopropanol, and from absolute ethanol freshly distilled from calcium oxide.

*Preparation of Dispersions.* Dispersions were prepared by extracting about 100 mg. of dried gluten or an equivalent amount of flour with 10 ml. of the dispersing agent in glass-stoppered test tubes. In most of the experiments the tubes were kept in a thermostat at 25°C. for 3 days and were shaken at regular intervals. Some experiments were carried out in which the tubes were placed in boiling water for 15 minutes.

The dispersions were separated from the residues by centrifuging at about 1,000 g. for 20 minutes and decanting the supernatants. The residues were then shaken up with 3–5 ml. of the dispersant, the tubes recentrifuged, and the supernatant decanted. This process was repeated and the three supernatants combined.

*Degree of Dispersion.* This was obtained from the amount of protein in dispersion and the amount of undispersed protein remaining in the residue. Suitable aliquots of the supernatants and the residues were analyzed by a semimicro-Kjeldahl procedure. Water was added to the supernatant aliquots to distill off the chloroethanol or alcohol and prevent the formation of volatile sulfates. Gluten residues were usually dissolved in 50% sulfuric acid. Very small gluten residues were digested *in toto*. All determinations were carried out in duplicate.

*Precipitated Gluten.* About 2 g. of gluten dispersed in 200 ml. of dispersant were precipitated by the addition of 200–400 ml. of ether. After standing for about 5 days, the clear supernatant was centrifuged off and a fresh portion of ether was added to the precipitate. The ether was removed after another 3–5 days and the precipitated acid gluten was dispersed in 200 ml. of water in a Waring Blendor. After the foam had settled, the gluten was reprecipitated by addition of 0.1*N* sodium hydroxide to pH 6.8. The precipitated gluten was then dried *in vacuo*.

*Amide Content.* This was determined by the 2*N* hydrochloric acid extrapolation method as described by Leach and Parkhill (11). No correction was made for free ammonia.

*Esterification.* The number of alkoxy groups per $10^5$ g. of protein were determined by the Zeisel method. These determinations were carried out by the CSIRO Microanalytical Service, Melbourne, in sixfold replication for each sample, and the results were averaged. Calculation of the degree of esterification was based on Fraenkel-Conrat and Olcott's (7) figure of 36 carboxyl groups per $10^5$ g. of original gluten protein. The blank due to the methionine content of the original gluten protein was subtracted from the number of alkoxy groups found in the precipitated gluten proteins (4,7).

*Viscosity.* Bulk viscosities were determined in B.S. No. 1 capillary viscometers. No kinetic energy correction was made.

*Surface Viscosity.* Surface viscosity measurements were carried out at the oil/water interface with an oscillating-needle, surface-torsion pendulum as described by Tschoegl and Alexander (16).

## Results

*Influence of Water and Hydrochloric Acid Content.* To find an explanation for the difference in gluten-dispersing power of unpurified and distilled chloroethanol, the influence of the two most likely impurities, namely water and hydrochloric acid, was investigated. Analysis of several laboratory-grade samples showed them to contain about 3–5% water, with acidities from 0.006 to 0.06*N*, or from 0.02 to 0.23% w/v expressed as hydrochloric acid.

Gluten was therefore dispersed in 0.01 and 0.001*N* solutions of hydrochloric acid in chloroethanol containing from 0 to 40% water. Figure 1 shows a plot of the degree of dispersion obtained as a function of the hydrochloric acid and water content of the choloroethanol.

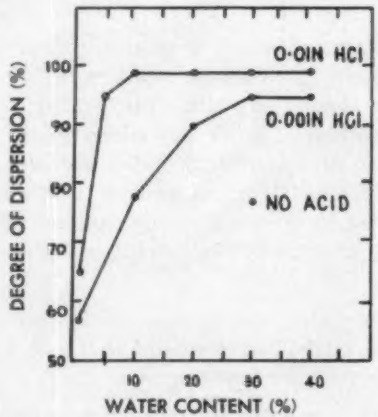


Fig. 1. Influence of water and hydrochloric acid content on degree of dispersion of gluten in chloroethanol.

In the 0.01*N* solution 95% dispersion was obtained with only 5% water and dispersion was virtually complete with 10% water; further increases in the water content had no significant effect. In the 0.001*N* solution, on the other hand, dispersion was only 95% with 40% water, although the same degree of dispersion was already reached with 30%. In carefully neutralized chloroethanol of 30% water content the degree of dispersion was only 77%.

In another experiment the influence of increasing amounts of hydrogen chloride in anhydrous chloroethanol was investigated. Table II shows that the degree of dispersion of gluten increased with the hydrogen chloride content, and complete dispersion was obtained

TABLE II
INFLUENCE OF HYDROGEN CHLORIDE CONTENT ON DEGREE OF DISPERSION OF GLUTEN IN ANHYDROUS CHLOROETHANOL

| Normality of Acid | Degree of Dispersion | Normality of Acid | Degree of Dispersion |
|---|---|---|---|
| 0.001 | 56.1 | 0.04 | 89.6 |
| 0.005 | 64.3 | 0.06 | 97.6 |
| 0.01 | 61.2 | 0.08 | 98.8 |
| 0.02 | 77.6 | 0.10 | 99.2 |

with about 0.08–0.10$N$ hydrogen chloride.

*Effect of Time on Degree of Dispersion.* Dispersion was most rapid in the 0.01$N$ aqueous acid chloroethanol solutions. It was usually complete within 24 hours and could be speeded up by more frequent shaking. Dispersion in 0.001$N$ aqueous solutions took a little longer, and was slowest in 0.1$N$ anhydrous solutions. The effect of time on the degree of dispersion in the latter was therefore studied in more detail. One hundred-milligram portions of gluten were shaken at regular intervals with 10 ml. of anhydrous 0.1$N$ acid chloroethanol. From time to time a tube was withdrawn and centrifuged, and the degree of dispersion was determined. Table III shows that the greatest increase in the degree of dispersion occurred during the first 24 hours (overnight) and that dispersion was virtually complete in 3 days.

TABLE III
EFFECT OF TIME OF EXTRACTION ON DEGREE OF DISPERSION OF GLUTEN IN ANHYDROUS ACID (0.1$N$) CHLOROETHANOL

| Hours | Degree of Dispersion | Hours | Degree of Dispersion |
|---|---|---|---|
| 7 | 1.5 | 47 | 95.0 |
| 15 | 58.8 | 66 | 98.0 |
| 24 | 88.4 | 72 | 98.0 |
| 31 | 91.0 | | |

*Other Anhydrous Acid-Alcohol Systems.* To see whether the combination of chloroethanol and hydrochloric acid was unique, another experiment was carried out using 0.1$N$ solutions of various acids in chloroethanol on the one hand, and in unchlorinated alcohols, on the other. The results are shown in Table IV.

No swelling at all occurred and very little protein was dispersed in the unchlorinated alcohols. In chloroethanol, sulfuric acid was about as effective as hydrochloric acid, although the gluten particles seemed to disintegrate somewhat more rapidly. Formic acid was not as effective as the mineral acids. Trichloroacetic acid, a protein pre-

TABLE IV

DEGREE OF DISPERSION OF GLUTEN IN VARIOUS ANHYDROUS ACID-ALCOHOL SYSTEMS

| Dispersant | | Protein Dispersed | |
|---|---|---|---|
| Acid | Alcohol | at 25° C. | In Boiling Water Bath |
| | | % | % |
| Hydrochloric, 0.1*N* | Ethanol (abs.) | 0.6 | ... |
| Hydrochloric, 0.1*N* | Isopropanol | 2.8 | ... |
| Sulfuric, 0.1*N* | n-Butanol | 7.3 | ... |
| Hydrochloric, 0.001*N* | Chloroethanol (anhyd.) | 56.1 | 70.6 |
| Hydrochloric, 0.1*N* | Chloroethanol (anhyd.) | 99.2 | 99.5[a] |
| Sulfuric, 0.1*N* | Chloroethanol (anhyd.) | 99.4 | 99.6[a] |
| Formic, 0.1*N* | Chloroethanol (anhyd.) | 81.6 | 87.0 |
| Trichloracetic, 0.1*N* | Chloroethanol (anhyd.) | 82.1 | ... |

[a] Dissolved.

cipitant in aqueous solution, increased the dispersion in chloroethanol slightly more than formic acid. Precipitation occurred only after the addition of water.

*Effect of Temperature.* The effect of temperature was not investigated in detail. However, some observations were made in experiments in which the tubes were placed in a boiling-water bath for 15 minutes. The degrees of dispersion obtained in this way with gluten in anhydrous chloroethanol containing different acids are shown in the last column of Table IV. Heating of the dispersions caused a significant increase in the degree of dispersion in case of the 0.001*N* hydrochloric acid (control) and the 0.1*N* formic acid solutions. With the mineral acids, 0.1*N* hydrochloric and 0.1*N* sulfuric, the increase may not have been significant but the gluten dispersed spontaneously without shaking. With the other two dispersions, the undispersed gluten particles did not disintegrate on shaking. No precipitation occurred on cooling. The dispersions remained clear, although of a slightly yellowish-brownish color. When gluten was heated with 10% sodium salicylate or with 0.01*N* hydrochloric acid, it dispersed more easily on shaking but did not disperse spontaneously.

*Particle Size and Agitation.* Particle size had a considerable effect in the case of the less effective dispersants, the larger particles usually taking longer to disperse. Comparable results could only be obtained by using dry gluten bolted through gauze of the same mesh size.

The effect of agitation was more noticeable with the more effective dispersants, such as 0.01*N* hydrochloric acid in aqueous chloroethanol, where an increase in the frequency of agitation shortened the time required for complete dispersion. Simple mixing instead of shaking, in the case of 0.1*N* hydrogen chloride in chloroethanol, lowered the degree of dispersion by a few percent.

*Precipitation.* Gluten dispersed in anhydrous 0.1*N* acid chloroethanol could be precipitated with diethyl ether, acetone, isopropanol, and n-butanol. Ether caused an immediate precipitation of about 97% of gluten dispersed at room temperature and about 95% of gluten dispersed at the temperature of boiling water. Observations made on these precipitations largely paralleled those made by Jaffe and De Coene (10) on the precipitation of zein dispersed in 70% aqueous alcohol. When the supernatant was decanted from the centrifuged precipitate immediately after precipitation, the precipitate darkened and formed a smeary, sticky gel. When left standing for several days under fresh portions of ether before centrifugation, the precipitate could be obtained in the form of a grayish powder. The material precipitated from aqueous redispersion, as described in the section on methods, showed no cohesion and resembled oat protein as obtained by the method of Cunningham *et al.* (5). A vacuum-dried sample of this material could not be redispersed in a 0.01*N* solution of hydrochloric acid in aqueous chloroethanol.

Addition of water to anhydrous acid chloroethanol dispersions produced very fine colloidal suspensions which were perfectly stable, no precipitation occurring after standing for several weeks. Dilution (tenfold) with virtually acid-free chloroethanol likewise produced no precipitation.

From dispersion in a 0.01*N* solution of hydrochloric acid in chloroethanol containing 30% of water, ether precipitated about 98% of the gluten in the form of a fine suspension which settled out on several (usually 5) days' standing to a clear supernatant. The material precipitated from aqueous redispersion resembled in every respect the wet gluten obtained by dispersion of dry gluten in dilute acid and precipitation by neutralization with dilute sodium hydroxide as described by Udy (17).

*Deamidation and Esterification.* Column 2 of the table below shows the percent loss in amide nitrogen relative to the original gluten, and column 3 the extent of esterification (percent of free

| *Sample* | *Loss in Amide N* | *Esterification* |
|---|---|---|
| | % | % |
| A | 0.4 | .. |
| B | 4.0 | 19 |
| C | 16.4 | 41 |

carboxyl groups esterified), for gluten recovered by precipitation with ether, redispersion in water, and reprecipitation by neutralization.

Sample A represents gluten recovered from dispersion in 0.01$N$ hydrochloric acid in aqueous (30%) chloroethanol. Samples B and C were recovered from dispersion in 0.1$N$ hydrogen chloride in anhydrous chloroethanol. Sample B was dispersed at room temperature and sample C at the temperature of boiling water.

No esterification and only an insignificant loss of amide nitrogen occurred in the aqueous chloroethanol. Some esterification and loss of amide nitrogen occurred in the anhydrous chloroethanol at room temperature. Temperature had a marked effect on both esterification and loss of amide nitrogen.

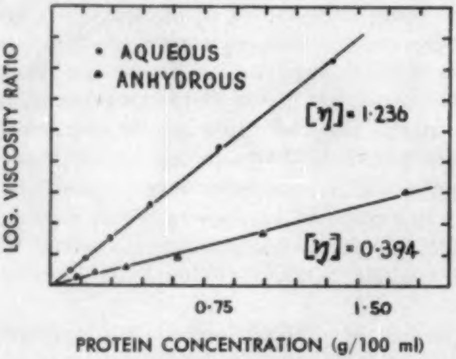


Fig. 2. Dependence of the logarithm of the viscosity ratio on gluten concentration. Disperson in aqueous (30%) acid (0.01$N$ HCl) and anhydrous acid (0.1$N$ HCl) chloroethanol.

*Bulk Viscosity Measurements.* Figure 2 shows the results of bulk viscosity measurements carried out on anhydrous (0.1$N$ HCl) and aqueous (30% water, 0.01$N$ HCl) acid chloroethanol dispersions of gluten. The logarithm of the viscosity ratio ($\eta/\eta_0$) is plotted against concentration. Since the regression lines pass through the origin, the limiting viscosity number (intrinsic viscosity) may be obtained simply from $[\eta] = 2.303b$ where b is the slope of the regression line. The limiting viscosity number was 0.394 in anhydrous acid chloroethanol and 1.236 in aqueous acid chloroethanol. Dispersions containing more than about 17–18 mg. per ml. were not obtainable.

*Surface Viscosity Measurements.* Figure 3 shows plots of the surface viscosity against time for films spread from anhydrous and aqueous acid chloroethanol. The curve obtained with gluten dispersed in 10% w/v sodium salicylate is included for comparison. The limiting surface viscosity (16) was 4.0 surface poises for films

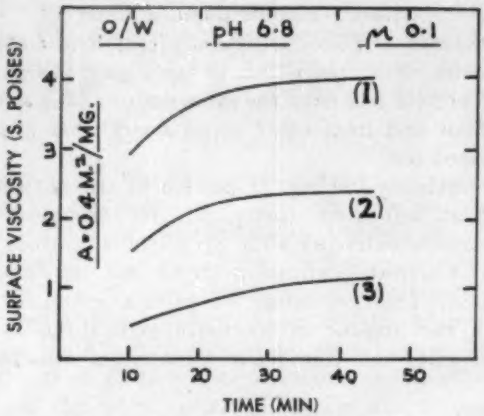


Fig. 3. Surface viscosity of gluten films spread from dispersion in anhydrous acid (0.1*N* HCl) chloroethanol (1), aqueous (30%) acid (0.01*N* HCl) chloroethanol (2), and 10% w/v sodium salicylate (3).

spread from dispersion in anhydrous acid chloroethanol, 2.4 surface poises for films from aqueous acid chloroethanol, and 1.4 surface poises for films from salicylate dipersions.

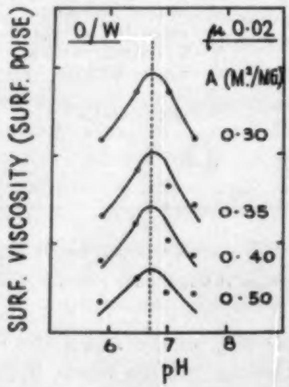


Fig. 4. Surface isoelectric point of gluten from films spread from dispersion in aqueous (30%) acid (0.01*N* HCl) chloroethanol.

Figure 4 shows a plot of the surface viscosity against the pH of the substrate at various areas per mg. protein for films spread from dispersion in aqueous acid chloroethanol. These plots show maxima

corresponding to a surface isoelectric point of about 6.7.

*Direct Extraction of Flours with Anhydrous Acid Chloroethanol.* Some experiments were carried out to investigate the possibility of extracting the protein free from the influence of added water directly from wheat flour and from other cereal flours from which glutens cannot be washed out.

Portions containing 100 mg. of protein of strong, medium, and weak wheat flour, and of rye, barley, oat, and carob-germ flour were therefore repeatedly extracted with 10 ml. of anhydrous 0.1$N$ acid chloroethanol. Complete extraction could not be obtained in a single operation. The percentage of protein extracted increased, however, with the number of successive extractions, as shown in Fig. 5, which gives the cumulative percentages achieved in four extractions.

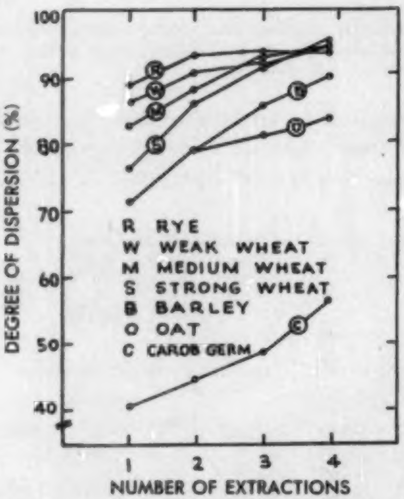


Fig. 5. Degree of dispersion of cereal flour proteins in anhydrous acid (0.1$N$ HCl) chloroethanol.

Extraction of about 95% of the wheat and rye flour proteins was attained in four extractions. Of the wheat flours the weak flour was most and the strong flour least rapidly extracted. This was confirmed in a repeat experiment. Rye flour behaved like a rather weak wheat flour. Extraction of barley (90%) and oat (84%) flours was less satisfactory, and only about 56% of the carob-germ flour could be extracted under the same conditions.

*Direct Extraction of Wheat and Oat Flour with Aqueous Acid*

*Chloroethanol.* By contrast, aqueous 0.01*N* acid chloroethanol extracted about 80% of wheat protein from the medium wheat flour and about 81% of oat protein in a single extraction, and about 91% of wheat protein and about 86% of oat protein in two extractions.

### Discussion

From the results presented here it appears that the chloroethanol used by Hess (9) could not have been free from acid and/or water. Only about 60% of gluten disperses in acid-free anhydrous chloroethanol. More gluten can be dispersed in the presence of hydrochloric acid, but this involves some deamidation and the esterification of part of the free carboxyl groups. These changes result in a shift of the isoelectric point, which was found to be about 7.5 by surface viscosity and surface pressure measurements (15,16). Whether the loss of cohesion in the reprecipitated gluten is due to esterification is difficult to say at this stage, since other possibilities such as the removal of lipids would have to be excluded.

When the chemical changes are either of little consequence or may be taken into account (e.g. in molecular weight determinations), anhydrous acid chloroethanol allows the examination of wheat and other cereal proteins free from the influence of added water. This dispersant may also facilitate examination by infrared spectroscopy which is difficult in the presence of water.

The esterification of gluten in 0.1*N* solutions of hydrogen chloride in various alcohols was investigated by Fraenkel-Conrat and Olcott (7), who found that esterification was complete only in methanolic solutions. Ethanol, propanol and n-butanol reacted more sluggishly, and hardly any esterification occurred in isopropanolic solutions. The esterification of gluten in chloroethanolic solution appears to be intermediate between that in propanolic and isopropanolic solution. It is interesting to note that the partly esterified gluten remains soluble in anhydrous acid chloroethanol, whereas it is apparently insoluble in the unchlorinated acid alcohols (7).

From a dispersion in aqueous acid chloroethanol, gluten may be recovered with its chemical and physical properties essentially unaltered. The isoelectric point was found to be about 6.7 by surface viscosity measurements (Fig. 4). It is worth noting that gluten dispersed virtually completely in a 0.01*N* solution of hydrochloric acid in 70% aqueous chloroethanol, whereas a similar ethanolic solution dispersed only about 72% of the gluten under identical conditions. The limiting viscosity number of gluten was 1.236 in aqueous acid chloroethanol and 0.394 in anhydrous acid chloroethanol. In 8%

sodium salicylate Udy (18) found the limiting viscosity number of gluten to be 0.309, while from the data of Geerdes and Harris (8) it appears to be about 0.200 to 0.250 in dilute acetic acid.

Gluten dispersed in either anhydrous or aqueous acid chloroethanol spreads readily at an interface. The limiting surface viscosities (16) from the aqueous dispersion are higher than those obtained from dispersion in sodium salicylate (cf. Fig. 3). Still higher limiting surface viscosities are obtained from the anhydrous dispersion. While the aqueous dispersion therefore yields higher limiting bulk viscosity numbers, the anhydrous dispersion gives higher surface viscosities. At the surface concentrations used, the surface viscosities are more of the nature of solid viscosities than solution viscosities. Further work is required to elucidate the difference in the limiting surface viscosities obtained from the two different dispersions.

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# EFFECT OF SOME VOLATILE CHEMICALS ON THE MICROBIAL SPOILAGE OF MOIST KAFIR CORN (Andropogon sorghum) UNDER AIRTIGHT STORAGE[1]

K. S. SRINIVASAN AND S. K. MAJUMDER[2]

## ABSTRACT

Kafir corn at 20% moisture was fumigated with methyl bromide and ethylene dibromide and hermetically stored for 60 days at 25°–29°C. Molds and bacteria were destroyed. Changes in fat acidity, water-soluble acidity, reducing sugars, amino nitrogen, and weight/volume ratio were retarded. Similar results were obtained with chloropicrin and ethylene oxide. The possibility of cold sterilization of moist grain and storage under aseptic conditions in hermetic storage is discussed.

The critical role of moisture in the deterioration of stored grain has been widely recognized. Kolkwitz (9), Swanson (24), Milner and Geddes (17,18) and Semeniuk *et al.* (22) have established the interaction of time, temperature, and moisture on the degree of deterioration of grains. Microorganisms are considered to be mainly responsible for the deterioration of moist grain, owing to their lipolytic, amylolytic, and proteolytic activities (5,15).

Kafir corn *(Andropogon sorghum)* is generally stored in underground pits in India. Although insect infestation is prevented through depletion of oxygen and accumulation of carbon dioxide under airtight storage (4), biochemical and microbiological degradation of certain kinds proceeds unhindered in the stored moist grain (7). The degree of spoilage in kafir corn stored in underground pits is quite high.

Several fungicidal agents (14,16,28) have been used to inhibit microbial activity on moist grain. Unfortunately, chemicals (14,16,24) such as thiourea, mercuric chloride, ceresan, and arasan, hitherto found suitable for the storage of seed grains, cannot be employed on food grains for toxicological reasons.

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Previous investigations of Majumder *et al.* (11,12) on different volatile organic chemicals have shown the usefulness of methyl bromide and a 3:1 mixture of ethylbromide-ethylenedibromide (v/v) for an initial reduction in microbial population and beneficial effects on subsequent storage. Matz and Milner (13) reported that a 1:1 mixture of propylene oxide and carbon tetrachloride was effective in reducing the deleterious changes which occur in damp grain; but the results obtained with this mixture suggested that it could be used as a short-term measure until the grain could be dried to safe moisture level. Majumder *et al.* (10) have used methyl bromide fumigation as an adjunct to drying chips of moist tapioca (*Manihot utillissima*) to safe moisture levels; the microbial count was brought down from an initial 125 million to 1,500 per g. at a dosage of 5 lb. per 1,000 cu. ft. and exposure period of 48 hours. Under the same conditions in wheat (25% moisture) the population was brought down from 2.4 millions to 720 per g.

In the present investigation, attempts have been made to study the deteriorative changes in moist kafir corn *(Andropogon sorghum)* under airtight storage and the effects of some fumigants such as ethylene dibromide, methyl bromide, chloropicrin, and ethylene oxide in preventing these undesirable changes.

## Materials and Methods

Freshly harvested kafir corn (variety Narkin) was dried to initial moisture of 11%. The samples were distributed in 700-g. lots in 1-liter wide-mouthed glass-stoppered bottles, and moisture contents were adjusted to 11, 15, 20, and 30% by adding the required amount of sterile water, mixing thoroughly, and shaking intermittently for 24 hours. The bottles were tightly stoppered (silicone grease used as lubricant), sealed with beeswax, and incubated at 30°C.

In a second experiment, ethylene oxide, ethylene dibromide, methyl bromide, and chloropicrin were used at dosages of 32, 64, and 96 mg. per liter on 10-kg. grain lots in properly clamped 16-liter desiccators. Fumigants ethylene oxide and methyl bromide were applied from a microburet at −10°C.; chloropicrin and ethylene dibromide were introduced into the desiccators at room temperature (26°C.). The moisture level of the grain was brought to 20% before the fumigants were introduced into the desiccators. The samples were analyzed for fat acidity, water-soluble acidity, reducing sugars, amino nitrogen, germination, microbial counts, fumigant residues, and organoleptic qualities at the end of storage period, 60 days.

The following methods were adopted for analysis of the samples: *Fat acidity* was determined according to the method of Zeleny and

Coleman (28). *Water-soluble acidity* was determined by the AOAC method (3). *Total reducing sugars* were estimated by the micro method of Somogyi (23). *Amino nitrogen* was determined by Samuel's method (21). *Weight/volume (w/v) ratio* was determined by measuring the displacement of water by the grain in a measuring cylinder.

*Total microbial counts* were determined on seeds ground aseptically in a laboratory grinder. The grinder was sterilized and the ground grains were sampled aseptically according to Teunisson (25). Counts were taken from colonies developed on potato-dextrose agar plates adjusted to pH 6.8 and incubated at 30°C. for 5 days. For anaerobic counts, serial dilution plates were incubated at 30°C. in desiccators containing alkaline pyrogallol. Methylene blue tests (20) indicated the absence of oxygen in the desiccators used for incubating plates for anaerobic counts.

*Moisture* was determined by the two-stage air-oven method (1) with ground samples at 130°C. for 1 hour. The results are reported on moisture-free basis.

*Germination* of kafir corn was ascertained by counting the number of germinated kernels on salt-free malt agar in Petri dishes after 5 days of incubation at 26°–29°C.

*Fumigant residues* were estimated for ethylene dibromide and methyl bromide according to the method of Young, Carter, and Soloway (27) after aeration of the samples in still air on shallow enameled trays at room temperature for 7 days. Residues of chloropicrin and ethylene oxide were not determined.

*Organoleptic qualities* of the samples were judged by a panel of judges on the aerated grains, flour and *roti* (conventional unleavened bread) by triangle difference test method (19). *Roti* was prepared by rolling 24 g. dough to a circle 4 in. in diameter and baking it on a hot plate until it attained a light brown color.

## Results and Discussion

At the end of 30, 90, and 150 days, kafir corn samples were analyzed for fat acidity, water-soluble acidity, reducing sugar, amino nitrogen, viability, microbial count, and weight/volume ratio. The results are presented in Table I.

These data indicate rapid increase with time of fat acidity, water-soluble acidity, reducing sugar, amino nitrogen, and microbial count in grains stored at 15, 20, and 30% moisture. There is an appreciable increase in the fat acidity values between 15 and 20% moisture as compared to values between 20 and 30% moisture. Water-soluble acidity of the samples, on the other hand, shows a progressive increase

TABLE I

BIOCHEMICAL AND MICROBIAL CHANGES IN STORED KAFIR CORN

(Average values of five replicates)

| Storage Period and Moisture Percentage | | Fat Acidity [a] | Water-Soluble Acidity [b] | Reducing Sugar | Amino Nitrogen | Germination | Microbial Count (per g. $\times 10^5$) | | Wt./Vol. Ratio |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Aerobic | Anaerobic | |
| | | mg/100g | mg/100g | mg/100g | mg/100g | % | | | |
| Control – | 11[c] | 31 | 63 | 160 | 10 | 96 | 1.1 | 0.6 | 0.81 |
| 30 days – | 11 | 40 | 84 | 186 | 12 | 93 | 1.9 | 2.0 | .79 |
| | 15 | 64 | 90 | 200 | 16 | 81 | 3.2 | 4.0 | .77 |
| | 20 | 144 | 112 | 310 | 20 | 56 | 5.4 | 8.4 | .75 |
| | 30 | 151 | 208 | 350 | 42 | nil | 9.4 | 10.0 | .74 |
| 90 days – | 11 | 54 | 124 | 189 | 12 | 90 | 2.4 | 3.7 | .78 |
| | 15 | 82 | 129 | 235 | 21 | 30 | 6.5 | 9.4 | .76 |
| | 20 | 169 | 148 | 385 | 26 | nil | 20.0 | 22.0 | .74 |
| | 30 | 170 | 288 | 490 | 70 | nil | 22.0 | 23.0 | .73 |
| 150 days – | 11 | 59 | 90 | 250 | 14 | 89 | 4.0 | 6.4 | .75 |
| | 15 | 94 | 115 | 270 | 25 | 6 | 12.0 | 44.0 | .73 |
| | 20 | 180 | 130 | 405 | 50 | nil | 25.0 | 54.0 | .72 |
| | 30 | 208 | 266 | 470 | 120 | nil | 18.0 | 74.0 | 0.69 |

[a] Milligrams of potassium hydroxide per 100 g.
[b] Milligrams of sodium hydroxide per 100 g.
[c] Analyses at the start of the experiment.

with time and proportional to the increase in moisture levels. Reducing sugars showed a progressive increase with time and moisture contents. At 20% moisture level and above, the germination percent was nil. With 15% moisture after 150 days of storage, germination percentage dropped as low as 6%. The total microbial count increased with the period of incubation. The anaerobic population was comparatively higher than the aerobic. There was considerable change in the density of the seeds at 30% moisture level on storage for 150 days. All the above data indicated that kafir corn stored hermetically at 11, 15, 20, and 30% moisture underwent biochemical and microbiological degradation. Lipolytic, amylolytic, and proteolytic activities were indicated by the changes in fat acidity, reducing sugars, and amino nitrogen respectively.

The data on the effects of fumigants – ethylene oxide, ethylenedibromide, methyl bromide, and chloropicrin used at dosages of 32, 64, and 96 mg. per liter on grain containing 20% moisture – are presented in Table II.

The results reveal that, in contrast to the changes in the control sample containing 20% moisture, the fumigants appreciably retarded adverse changes in treated moist grains. The criteria for the deteriorative changes in grains fumigated with methyl bromide, chloropicrin, and ethylene oxide maintained almost the same values as the control

TABLE II

EFFECT OF CERTAIN VOLATILE CHEMICALS ON THE DETERIORATIVE CHANGES IN KAFIR CORN

(Average values of five replicates)

Moisture, 20%; period of storage, 2 months; temperature, 25°–29°C.

| Chemical | Fat Acidity | Water-Soluble Acidity | Reducing Sugars | Amino Nitrogen | Microbial Count (per g. $\times 10^5$) | | Fumigant Residues | Organoleptic Quality[c] |
|---|---|---|---|---|---|---|---|---|
| | | | | | Aerobic | Anaerobic | | |
| | mg/100g | mg/100g | mg/100g | mg/100g | | | ppm Br | |
| Control, 11%M[a,b] | 28 | 63 | 165 | 10 | 1.2 | 0.5 | .. | G |
| Control, 20%M[a] | 150 | 115 | 320 | 25 | 20 | 21 | .. | MS |
| Ethylene dibromide, mg/l. | | | | | | | | |
| 32 | 56 | 94 | 260 | 15 | 4.4 | 3.6 | 12 | FO |
| 64 | 38 | 77 | 180 | 13 | 0.6 | 0.2 | 22 | SF |
| 96 | 28 | 70 | 170 | 12 | 0.2 | 0.1 | 23 | A |
| Methyl bromide, mg/l. | | | | | | | | |
| 32 | 41 | 76 | 210 | 14 | 1.0 | 0.3 | 15 | SF |
| 64 | 32 | 69 | 182 | 14 | 0.1 | ... | 23 | A |
| 96 | 28 | 68 | 175 | 11 | ... | ... | 27 | A |
| Chloropicrin, mg/l. | | | | | | | | |
| 32 | 42 | 80 | 214 | 15 | 1.1 | ... | .. | SF |
| 64 | 33 | 72 | 183 | 14 | 0.5 | ... | .. | A |
| 96 | 28 | 69 | 169 | 12 | ... | ... | .. | A |
| Ethylene oxide, mg/l. | | | | | | | | |
| 32 | 39 | 74 | 199 | 15 | 0.6 | ... | .. | SO |
| 64 | 36 | 66 | 182 | 12 | ... | ... | .. | A |
| 96 | 30 | 64 | 171 | 11 | ... | ... | .. | A |

[a] Analyses at the start of the experiment. (M, moisture.)
[b] Germination in control, 11% moisture, 96%; otherwise, none.
[c] Dough and unleavened bread. G, good; MS, musty; FO, fermented odor; SF, slightly fermented; A, acceptable; SO, slight odor.

sample at 11% moisture. Although ethylene dibromide retarded microbial activity, absolute sterilization of the grain could not be achieved even at a dosage of 96 mg. per liter under the experimental conditions. Surface mycelial growth was visible in top layers in the grain samples fumigated with ethylene dibromide within 30 days of incubation. Methyl bromide and chloropicrin at 96 mg. per liter and ethylene oxide at 64 mg. per liter brought down the microbial population to zero under sustained fumigation for the whole incubation period of 60 days. Owing to sterilization of the grain at these concentrations by the fumigants, biological decomposition was retarded. The organoleptic tests indicated the soundness of the hermetically stored moist grain fumigated with methyl bromide, chloropicrin, and ethylene oxide, whereas the control sample was unacceptable. Usually a characteristic taint develops in wheat flour fumigated with methyl bromide

(26), but no off-odor could be detected by the test panel on the kafir corn samples experimentally fumigated.

Grain may be stored satisfactorily in metal and concrete silos in temperate climates. In the tropics, however, wide fluctuations in diurnal temperature, high average temperature, translocation of moisture, and sweating accelerate the rate of microbiological and biochemical degradation. This investigation indicates the possibility of using volatile sterilizing agents for suppressing deteriorative changes and preventing external microbial contamination during storage.

Methyl bromide, ethylene oxide, and chloropicrin were the best fumigants for sterilizing the grain under hermetic storage. Further information on the residues and their effects on the various chemical constituents of the grain under such high dosages and exposure periods will be required. In the present study, the residues of methyl bromide were within the permissible limits as prescribed in Canada (6) and the United States (2) for cereals. Precise information is available on the toxicological aspects of the residues and reaction products of methyl bromide (26) and ethylene oxide (8), but chloropicrin requires critical studies on the residues and toxicological aspects for its use under these conditions.

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# EFFECT OF PARBOILING ON THE THIAMINE, RIBOFLAVIN, AND NIACIN CONTENTS OF WHEAT[1]

Z. I. SABRY AND R. I. TANNOUS


## ABSTRACT

Samples of the hard wheat, variety Senator Capelli, were parboiled for 0.5-, 1-, 1.5-, 2-, and 3-hour periods. The samples were then dried and crushed, and the bran was removed. The effect of the various heat-treatments on the thiamine, riboflavin, and niacin contents was determined in the crushed parboiled grain as well as in the bran. The longer the parboiling time, the more thiamine was destroyed. While the riboflavin content of wheat was hardly affected by parboiling and subsequent oven-drying, a substantial amount of the vitamin was destroyed when the product was sun-dried. The niacin level tended to increase with the increase in parboiling time. These changes appear to be due to direct heat destruction and the redistribution of the vitamins from the outer layers to the inner layers of the wheat grain.


Parboiled wheat is one of the most important cereal products in the Middle East. It is known as *burghul* in the Arab countries and as *bulgur* in Turkey and Central Europe.

Parboiled wheat is usually prepared on a small scale in the villages. After the wheat is separated from the chaff, it is washed in cold water and boiled in an uncovered copper kettle for 2 to 3 hours. The boiled wheat is spread in the sun to dry and then is usually moistened with a sprinkle of water before it is crushed and the bran removed.

Saracoglu (3), Saracoglu and Babadag (4), and Shammas and Adolph (5) found that thiamine and, to a greater extent, riboflavin decreased considerably during the parboiling process. However, the niacin content of the parboiled product was found to be slightly higher than that of wheat (5).

The present work is an attempt to determine the extent of heat-treatment necessary for the parboiling process and its effect on the level of thiamine, riboflavin, and niacin in the wheat grain as well as in the bran.

## Materials and Methods

*Preparation of Samples.* Seven 1-kg. samples were prepared from the hard wheat, variety Senator Capelli. An unboiled sample served as a control while five samples were boiled for 0.5-, 1-, 1.5-, 2-, and 3-

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hour periods. Water was added in such an amount that all of it was absorbed by the time boiling was checked in order to eliminate leaching of water-soluble nutrients. The amounts of water added per kg. of wheat were 950, 1,600, 2,100, 2,800, and 3,400 ml. for the boiling periods of 0.5, 1, 1.5, 2, and 3 hours, respectively. The boiled samples were spread on trays and dried in a forced-draft dehydrator. The dried samples were sprayed with a little water, then ground in a Wiley mill and left on trays overnight to dry at room temperature. The ground samples were sieved in a forced draft to remove the bran. The seventh sample received a 2-hour boiling treatment and was dried in the sun for 3 days. This was done to study the effect of sun-drying on the riboflavin content of the parboiled product.

*Assay Methods.* The thiochrome method (1) was used for the determination of thiamine. Riboflavin was determined by the fluorescence method (1). Niacin was assayed by a microbiological technique (1) using *Lactobacillus arabinosus* ATTC 8014[2] as the test organism.

### Results and Discussion

All the parboiled samples were rated acceptable with regard to color, texture, taste, and odor. The sample which received the 3-hour boiling treatment was slightly dark in color and showed moderate clumping and stickiness; that with 0.5-hour boiling had less gelatinization than the other samples. The uniformity in the gelatinization of the starch throughout the endosperm was judged by a translucent appearance and absence of opaque spots. The amount of bran separated from the parboiled grain decreased with the increase in boiling time during the first hour. The bran yields corresponding to 0, 0.5, 1, 1.5, 2, and 3 hours of boiling time were 16.5, 15.1, 14.4, 14.1, 14.0, and 14.2%, respectively. This difference in bran yields may be due to the gelatinization of starch in the endosperm during boiling and the trapping of part of the embryo and the scutellum into the parboiled product.

It is evident from the data expressed in Fig. 1 that the heat-treatment has a direct destructive effect on thiamine. The riboflavin content of the wheat grain decreased slightly as a result of the parboiling process, as shown in Fig. 2. However, when the parboiled wheat was dried in the sun, the loss of riboflavin amounted to 76% as compared with 20% loss in a sample receiving the same parboiling treatment but dried in the oven. The parboiling process brought about an increase in the niacin content of bulgur, apparently at the expense of the bran fraction content, as indicated in Fig. 3.

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[2] Obtained from the American Type Culture Collection, 2029 M Street N.W., Washington 6, D. C.

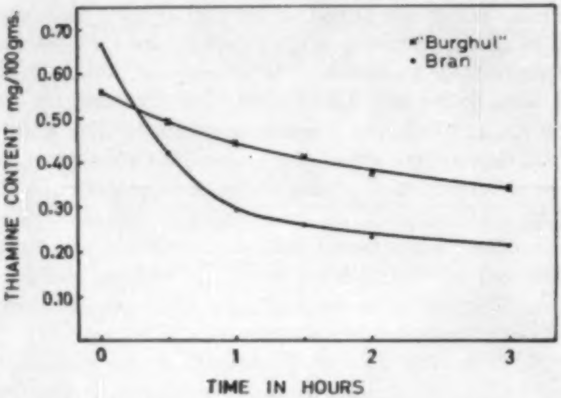


Fig. 1. Effect of parboiling on thiamine content of wheat.

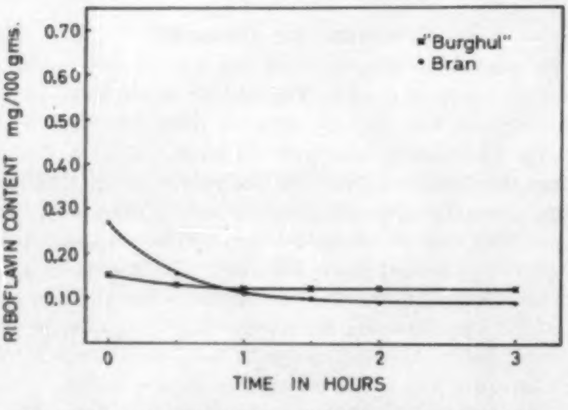


Fig. 2. Effect of parboiling on riboflavin content of wheat.

In all cases, it was found that the thiamine, riboflavin, and niacin levels decrease at a much faster rate in the bran than in the grain. This may be attributed to direct heat destruction as well as to redistribution of these vitamins from the outer layers to the inner layers of the wheat grain. Since the bran is rich in niacin and the germ is the part considered richest in thiamine and riboflavin (2), the vitamin redistribution has a more pronounced effect on the niacin content of the parboiled wheat than on the thiamine and riboflavin contents.

From the foregoing evidence, a parboiling time of 0.5 to 1 hour may be recommended for the making of bulgur. This would result in high-

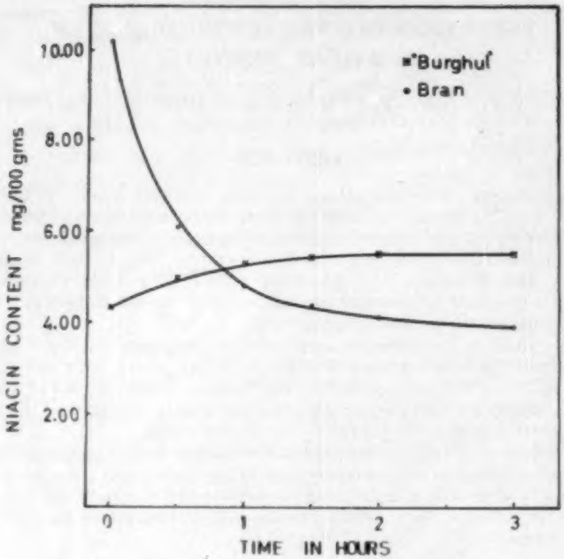


Fig. 3. Effect of parboiling on niacin content of wheat.

er retention of thiamine, while permitting almost the same increase in niacin content as is obtained with the conventional 2- to 3-hour boiling. Oven-drying of the product results in markedly better retention of riboflavin than does sun-drying, but economic considerations preclude oven-drying as a substitute for the preparation of bulgur, as at present, on a family or village basis.

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# DIFFUSION COEFFICIENTS OF WATER IN WHEAT KERNELS[1]

LIANG-TSENG FAN[2], DO SUP CHUNG[2], AND JOHN A. SHELLENBERGER[3]

## ABSTRACT

The diffusion coefficients of four varieties of wheat, Ponca (HRW) and Vernum (durum) wheats representing hard wheats and Seneca (SRW) and Brevor (SWW) wheats representing soft wheat, were measured under previously untried conditions (maximum temperature, 98.3°C. and maximum diffusion time, 8 hours). The theoretical treatment and the experimental techniques proposed by previous investigators were found to be valid under the conditions of the present investigation.

The values of the diffusion coefficients ranged from $2.7 \times 10^{-8}$ $cm^2/sec$ (26.7°C.) to $245.6 \times 10^{-8}$ $cm^2/sec$ (98.3°C.) for Ponca wheat, $2.2 \times 10^{-8}$ $cm^2/sec$ (30°C.) to $75.2 \times 10^{-8}$ $cm^2/sec$ (86°C.) for Vernum wheat; $3.1 \times 10^{-8}$ $cm^2/sec$ (26.7°C.) to $140.9 \times 10^{-8}$ $cm^2/sec$ (98.3°C.) for Seneca wheat; and $2.7 \times 10^{-8}$ $cm^2/sec$ (30°C.) to 89.2 $cm^2/sec$ (86°C.) for Brevor wheat.

The effective surface moisture content is independent of temperature, and the diffusion coefficients at temperatures below about 65°C. are practically independent of variety. The diffusion coefficients, and therefore the activation energies for diffusion, increase sharply above this temperature for four varieties of wheat.

The diffusion coefficient of water in wheat kernels is of practical importance in the drying and tempering of wheat, since these processes involve the transport of water in and out of wheat kernels.

Although the problem of water diffusion into wheat kernels has been studied for some time, most of the previous studies have been qualitative in nature. The study of mechanism and the quantitative treatment of data of water diffusion into wheat kernels, however, have been initiated only recently (1,2,3,5). Grosh and Milner (5) investigated the mechanism of water diffusion by visual and X-ray methods. They found that crack formation precedes the diffusion of water at the initial stage. Becker and his co-workers (1,2,3) developed a mathematical model based on Fick's Law of diffusion which correlates both absorption and desorption data.

The diffusion coefficients of water in four varieties of wheat, Ponca and Vernum representing hard wheat, and Seneca and Brevor representing soft wheat, measured at various temperature levels, are reported in this paper. The maximum temperature of 98.3°C. at which the measurement was made was considerably higher than that of 70.5°C. attained in previous measurements (1,2).

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### Theoretical Aspect

The diffusion coefficient is defined by Fick's Law as (6):

$$D\left(\frac{\partial^2 c}{\partial x^2}+\frac{\partial^2 c}{\partial y^2}+\frac{\partial^2 c}{\partial z^2}\right)=\frac{\partial c}{\partial \theta} \quad (1),$$

where D is the diffusion coefficient, c is the concentration of diffusing substance at a point in a solid, x, y, and z are Cartesian coordinates of the point under consideration, and $\theta$ is the diffusion time. Becker (1) demonstrated that equation 1 is integrated for the particle of arbitrary shape under the assumption that the surface-to-volume ratio, S/V, remains constant as:

$$\overline{C}=1-\frac{2}{\sqrt{\pi}}X+BX^2 \quad (2),$$

where $\overline{C}=\frac{\bar{c}-c_s}{c_0-c_s}$ in which $\bar{c}$ is the average concentration, $c_o$ the initial concentration, and $c_s$ the concentration at bounding surface, $X = S/V\sqrt{D\theta}$, and B = a dimensionless constant. If the concentration term is represented by the moisture content on a weight basis:

$$\overline{C}=\overline{M}=\frac{\overline{m}-m_s}{m_o-m_s} \quad (3),$$

in which $\overline{m}$ is the average moisture content at the given immersion time, $m_s$ is the effective surface moisture content, and $m_o$ is the initial moisture content (moisture contents expressed in g/g). Equation 2, then, can be written as follows:

$$\overline{M}=1-\frac{2}{\sqrt{\pi}}X+BX^2 \quad (4).$$

For small values of X, equation 4 approximates to:

$$1-\overline{M}=\frac{2}{\sqrt{\pi}}X \quad (5),$$

or, in terms of experimental variables:

$$\overline{m}-m_o=K\sqrt{\theta}$$

$$\text{where } K=\frac{2}{\sqrt{\pi}}(m_s-m_o)\frac{S}{V}\sqrt{D} \quad (6).$$

Becker (1,2) demonstrated that this equation was valid up to a moisture level of 0.57 g/g in wheat kernels.

### Materials and Methods

The varieties of wheat used in this study were Ponca (hard wheat), Vernum (hard wheat), Seneca (soft wheat), and Brevor (soft wheat). The pycnometer method was applied to measure the initial densities of wheat. A 50-cc. pycnometer with toluene as the fluid was used with

5-g. samples of wheat. Densities of Ponca, Vernum, Seneca, and Brevor wheats used were 1.357, 1.315, 1.312, and 1.345 g/cc respectively. Protein contents of Ponca, Vernum, Seneca, and Brevor were 12.7, 13.85, 10.5, and 9.63% respectively.

The initial moisture contents were 15.6% (dry basis) for Ponca, 14.72% (dry basis) for Vernum, 13.4% (dry basis) for Seneca, and 11.05% (dry basis) for Brevor.

To estimate the sphericity, the porosity was calculated by using the bulk and true densities of wheat. After the porosity was known, sphericity was estimated from the plot of sphericity as a function of porosity (4). The estimated sphericities were approximately 0.91 for Ponca, 0.90 for Vernum, 0.84 for Seneca, and 0.91 for Brevor. The values of the sphericity of Ponca, Vernum, and Brevor were identical with that of Thatcher wheat estimated by Becker (1,2).

The volume-to-surface-area ratio was then evaluated by using the formula (1), $V/S = \psi\, r_v/3$, where $\psi$ is the sphericity, and $r_v$ is the radius of a sphere with volume equal to that of the wheat kernel. The values of the volume-to-surface-area ratio, V/S, for Ponca, Vernum, Seneca, and Brevor wheats were found to be 0.0547, 0.0607, 0.0504, and 0.0597 cm. respectively.

Twenty-gram samples of wheat were placed in wire gauze baskets and immersed in a stirred water bath controlled within 0.5°C. of the set temperature. The pH of the steeping water used in this study ranged from 7.5 to 8.0. Experiments were carried out with temperatures ranging from 26.7° to 98.3°C., and for immersion periods from several minutes to 8 hours. At the end of each immersion period, each sample was quickly removed from the water bath and superficially dried on a large filter paper (2). After the surface water on the wheat kernels was removed, the weight of the sample was determined.

To evaluate the effective surface moisture content, samples were prepared with initial moisture contents ranging from 15.6 to 52.4% (dry basis) for Ponca, from 14.7 to 38.4% (dry basis) for Vernum, from 13.4 to 42.7% (dry basis) for Seneca, and from 11.05 to 34.5% for Brevor. Samples were immersed in water at 30° and 70°C. and their final moisture values were measured after 15 minutes.

## Results and Discussion

The relation between moisture gain and the square root of the immersion period is shown in Fig. 1 for Vernum wheat. Results for other wheats were similar to those of Fig. 1 for Vernum wheat. There was a rapid moisture pick-up by capillary action at the moment wheat kernels contact water. This moisture pick-up by capillary

action, $m_i$, increased slightly and linearly with increasing temperature. The increase was approximately 2% at 30° and 4% at 90°C. for each variety of wheat. As indicated in Fig. 1, $m_i$ was subtracted from the total moisture gain since $m_i$ was due to a phenomenon other than diffusion (2).

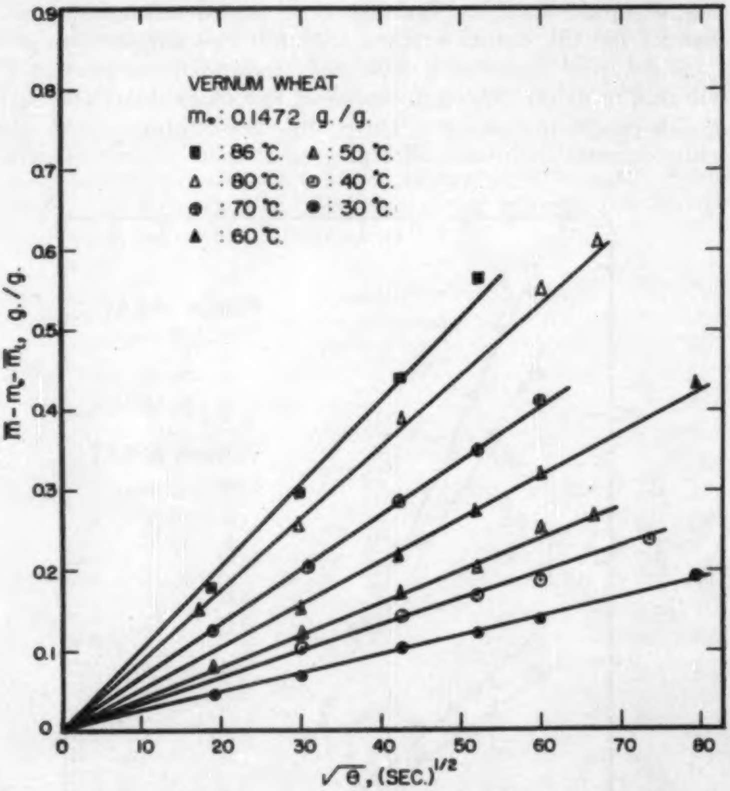


Fig. 1. Linear relation between moisture gain and square root of absorption time for Vernum wheat.

Ranges of validity of equation 6 were different for hard wheat and soft wheat. It was smaller for the latter, especially at high temperatures. The slopes of curves in Fig. 1 and similar figures for other wheats were obtained by the method of least squares.

The values of effective surface moisture content, $m_s$, required in evaluating the diffusion coefficients were measured at two temper-

ature levels as shown in Fig. 2 for Vernum and Ponca wheat (2). A similar figure was obtained for Seneca and Brevor wheat. The effective surface moisture content for Ponca wheat was in close agreement with that for Thatcher wheat as measured by Becker (2). This result indicates that the effective surface moisture content is, for all practical purposes, independent of temperature level. These values of effective surface moisture contents, which are equilibrium moisture contents for the diffusion process, should also indicate that the applicability of equation 5 or 6 above those moisture contents is empirical in nature. The applicability of Fick's Law ceases above the equilibrium moisture content. The equilibrium moisture content may actually change continually with progress of water absorption owing

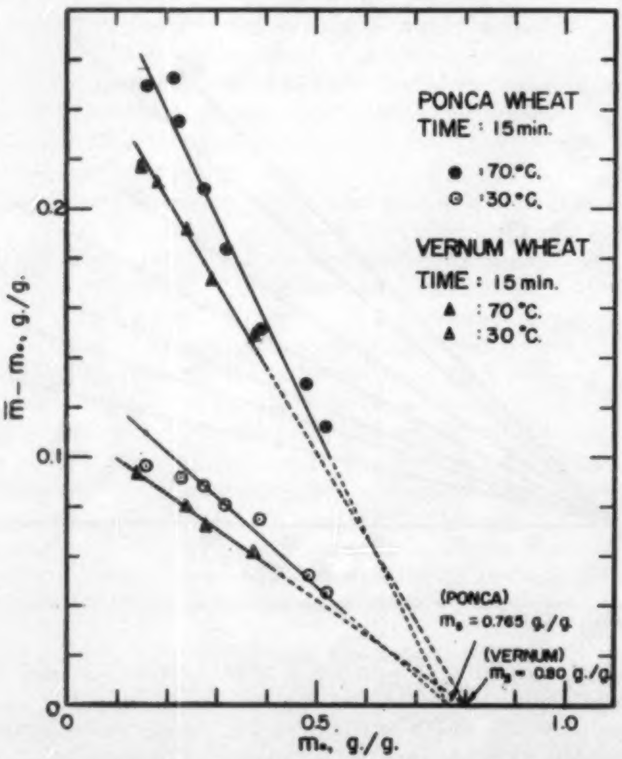


Fig. 2. Moisture gain as a function of initial moisture content at 70° and 30°C., showing the linear regression on effective surface moisture contents for Ponca and Vernum wheat.

to the considerable structural change of wheat kernels.

The diffusion coefficients, D, could be evaluated from equations 5 and 6 after the effective surface moisture contents were known.

$$D = \left[ \frac{\sqrt{\pi}}{2} \frac{V}{S} \frac{K}{(m_s - m_o)} \right]^2$$

The diffusion coefficients obtained are listed in Table I. Figure 3 for Vernum and Brevor wheat shows the diffusion coefficients calculated from experimental data as a function of the reciprocal of absolute temperature. A similar figure was also obtained for Ponca and Seneca wheat. These figures show Arrhenius relation for the diffusion coefficients, $D = D_o \exp\left(-\frac{E}{RT}\right)$, in which E is the activation energy, and $D_o$ is the constant. The constant (frequency factor), $D_o$, and the slope, (E/R), of the linear regression of the Arrhenius relation were estimated by the method of least squares.

TABLE I
DIFFUSION COEFFICIENTS

| TEMPERATURE | VARIETY OF WHEAT | | | |
|---|---|---|---|---|
| | Ponca | Vernum | Seneca | Brevor |
| °C | $D \times 10^8$ $cm^2/sec$ | $D \times 10^8$ $cm^2/sec$ | $D \times 10^8$ $cm^2/sec$ | $D \times 10^8$ $cm^2/sec$ |
| 26.7 | 2.7 | | 3.1 | |
| 30.0 | | 2.2 | | 2.7 |
| 32.2 | 4.4 | | | |
| 37.8 | 6.0 | | 6.0 | |
| 40.0 | | 5.0 | | 5.3 |
| 48.9 | 10.3 | | 12.8 | |
| 50.0 | | 7.2 | | 8.2 |
| 54.4 | 13.8 | | | |
| 60.0 | 18.2 | 14.6 | 22.3 | 16.3 |
| 65.6 | 27.5 | | | |
| 69.4 | | | 30.0 | |
| 70.0 | 41.1 | 23.3 | | 26.2 |
| 76.7 | 75.4 | | | |
| 80.0 | | 48.1 | | 53.8 |
| 81.7 | 118.6 | | | |
| 82.2 | | | 61.8 | |
| 86.0 | | 75.2 | | 89.1 |
| 98.3 | 245.6 | | 140.9 | |

It can be seen that a poor correlation by the Arrhenius relation results when the diffusion coefficients are correlated in a single linear regression for each variety of wheat. D at high temperature deviates markedly from a single regression. Careful inspection indicates that every plot of the Arrhenius relation shows consistently concave curvatures. The best linear correlation, however, is obtained when the

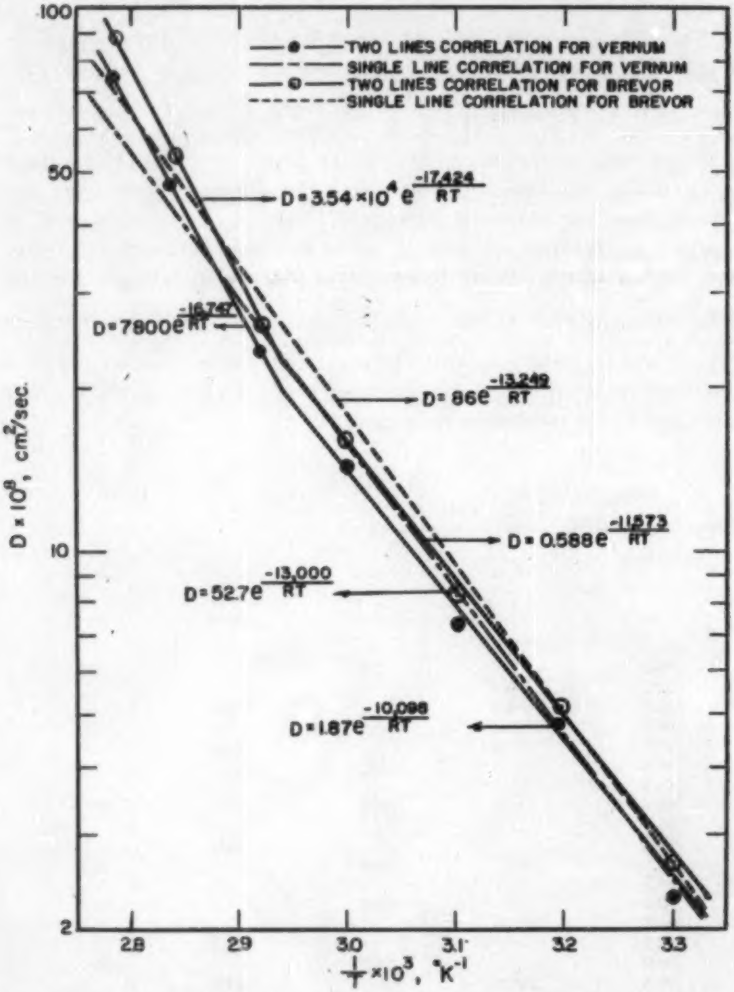


Fig. 3. Diffusion coefficient as a function of the reciprocal of absolute temperature for Vernum and Brevor wheat.

data are divided into two parts as shown in Fig. 3.

Activation energies, E, and constants, $D_0$ in a single regression line and two regression lines of the Arrhenius relation are presented in Tables II and III.

TABLE II

THE CONSTANTS, $D_o$, AND ENERGIES OF ACTIVATION, E, IN SINGLE LINE CORRELATION OF THE ARRHENIUS RELATION

| Variety of Wheat | $D_o$ | E |
|---|---|---|
| | $cm^2/sec$ | kcal/mol |
| Ponca | 142 | 13.3 |
| Vernum | 52.7 | 13 |
| Seneca | 6.3 | 11.4 |
| Brevor | 86 | 13.3 |

TABLE III

THE CONSTANTS, $D_o$, AND ENERGIES OF ACTIVATION, E, IN TWO-LINE CORRELATION OF THE ARRHENIUS RELATION

| Variety of Wheat | Range of Temperature | $D_o$ | E | $\Delta E$ |
|---|---|---|---|---|
| | C° | $cm^2/sec$ | kcal/mol | % |
| Ponca | Below 65 | 4.7 | 11.2 | 71.0 |
| | Above 65 | $5.7 \times 10^5$ | 19.2 | |
| Vernum | Below 65 | 1.9 | 10.1 | 63.2 |
| | Above 65 | $7.8 \times 10^3$ | 16.5 | |
| Seneca | Below 65 | 1.0 | 10.2 | 39.3 |
| | Above 65 | 390 | 14.3 | |
| Brevor | Below 65 | 0.59 | 11.6 | 50.6 |
| | Above 65 | $3.5 \times 10^4$ | 17.4 | |

The diffusion coefficients and activation energies at temperatures below about 65°C. fall within the range of the diffusion coefficients and the activation energy evaluated by Becker (2) for Thatcher wheat. The diffusion coefficients at temperatures below this are almost independent of variety of wheat in contrast to Fraser and Haley's results (2).

The activation energies at temperatures above 65°C., however, markedly increase for each variety of wheat. The increase is from 40 to 70% of the values below 65°C., with hard wheats generally showing greater increase than the soft wheats.

It appears that the rise in the value of E may be associated with thermal change of the molecular structure of wheat, specifically due to the starch gelatinization and protein denaturation (3; and personal communication from H. A. Becker). Gross changes in the macro structure which would affect the diffusion properties also could cause the change in activation energies. Gelatinization of starch and

protein denaturation are complex problems. In fact, there is still no clear picture of the mechanism of granule gelatinization and protein denaturation. It can be expected, however, that the gelatinization of starch and protein denaturation take place rapidly above 65°C.

**Acknowledgment**

The authors are grateful for various suggestions by M. Milner and G. M. Grosh in conducting this research.

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# INDEX TO VOLUME XXXVIII

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## SUBJECTS

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## CONTENTS

### Number 1, January, 1961



### Number 2, March, 1961

NUMBER 3, MAY, 1961

NUMBER 4, JULY, 1961



NUMBER 5, SEPTEMBER, 1961